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From the Institute of Biological Chemistry, University of Copenhagen, Denmark

Active Sodium and Chloride Transport in the Single Celled Marine Alga Halicystis Ovalis

By

ROBERT W. BLOUNT[1] and BLAINE H. LEVEDAHL[2]

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Abstract

BLOUNT, R. W. and B. H. LEVEDAHL. *Active sodium and chloride transport in the single celled marine alga Halicystis ovalis.* Acta physiol. scand. 1960. *49.* 1—9. — The active transports of sodium and chloride ions, between the vacuole and environmental solutions, were measured in the giant coenocyte of the marine alga *Halicystis ovalis.* Ion fluxes, determined isotopically, of individual single cells were measured by the short-circuit technique of USSING and ZERAHN (1951). Concentric pipettes were used to replace the vacuole sap with sea-water and to short-circuit the vacuole potential difference to zero. The mean net efflux of sodium represented 39.2 %, S. D. = 5.4, and the mean net influx of chloride 57.6 %, S. D. = 5.3, of the current flowing through a short-circuited cell. Therefore a summation of the current carried by the two net active fluxes can account for the total short-circuit current. Micro-electrode penetration of the protoplasm of the cell indicated that the potential difference of the protoplasm was identical with the vacuole potential; that the total potential difference develops at the outer membrane. A theory is presented which evaluates the effect of these transport systems on the total potential difference and on the osmotic stability of the cell relative to its environment.

The coenocyte of the marine alga *Halicystis ovalis* is nearly spherical in shape and in some cases attains a diameter of one centimeter. A large sap-filled vacuole represents the largest fraction of the cell's total volume. Enclosing the vacuole is the protoplasm which is about 10μ thick, multinucleate and

[1] Public Health Service Research Fellow of the Institute of Neurological Diseases and Blindness.

[2] Dept. of Zoology, University of California, Los Angeles.

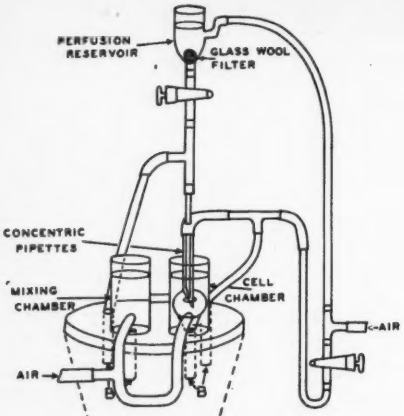

Fig. 1. Diagram of the apparatus used to perfuse the vacuole, detect and short-circuit the P. D. across the protoplasm. B indicates sea-water agar bridges.

contains many chloroplasts. In close contact with the protoplasm is an outer cellulose wall. When a glass capillary is inserted into the vacuole the cell forms a high electrical resistant seal around the capillary.

The sap is an inorganic salt solution of approximately the same ionic strength as its environmental sea-water, but the concentrations of most ions are different in the two solutions and the sap has a negative electrical potential relative to sea-water. Evaluation of the electro-chemical potential of the major monovalent ions in the two phases suggested that sodium and chloride were actively transported ions. Since this coenocyte is so large, concentric pipettes were introduced into the vacuole and the sap was replaced with sea-water. Thus, with identical solutions inside and outside the cell and with the vacuole potential shunted to zero, a short-circuit analysis for the active transport of sodium and chloride was made.

Methods

A diagram of the apparatus used in these experiments is seen in Fig. 1. The chamber assembly was composed of two lucite cylinders connected together by a straight lucite tube and a U-shaped glass tube; its volume was 4.5 ml. With both cylinders and U-tube filled with solution, a continuous circulation was brought about by forcing air into the

left-hand arm of the U-tube. The purpose of two chambers was to achieve good stirring without bubbling directly into the chamber containing the impaled cell. The mixing time was less than one minute.

The two chambers were fused to the top of a lucite cap which fitted onto a silver-silver chloride half cell assembly below. This assembly consisted of a lucite cylinder partitioned into four chambers and fused at its bottom to a rectangular base which was bolted to the stage of a vertical rack and pinion.

Vacuole perfusion

Perfusion was effected with concentric pipettes which had tip diameters of about 0.25 mm and 0.1 mm. The tip of the outer pipette was bevelled to an angle of 45° and to a sharp point with fine carborundum powder. The inner pipette was inserted through the wall of a short length of gum rubber tubing affixed to the outer pipette, and a simple thrust located this capillary into the bore of the outer pipette. The free end of the inner pipette was connected to a T-shaped tube; one branch connected this pipette to a silver-silver chloride half cell and the other, through a stopcock, to the perfusion reservoir. The outer pipette was similarly connected through a T-shaped tube to a silver-silver chloride half cell and through a stopcock to the perfusion reservoir. With the pipettes implanted in an alga, a continuous circulation of the vacuole was brought about by forcing air in at the air input T-tube. The volume of the pipette assembly was 3.5 ml; its rate of circulation was limited by the bore of the pipettes and was governed in its working range by the two stopcocks. The average mixing time was six minutes.

Electric circuits

The concentric pipettes served as electrodes for potential difference (P. D.) measurement and short-circuiting, as well as for the perfusion of the vacuole. The inner pipette and the cell chamber, by means of sea-water agar bridges, were connected through half cells to a high impedance voltmeter. Similarly, the external pipette and the chamber solution were connected to a shunt network. This circuit is identical to that described by Ussing and Zerahn (1951), and serves as a shunt of infinite conductance between vacuole and environment. The current flowing through the cell at zero P. D. was recorded by a galvanometer in the shunt circuit. Since sea-water is a relatively concentrated salt solution, errors due to flow potentials did not result from the dual function of the pipettes. Also, the large conductance of sea-water, compared with the membrane conductance of the cell, permitted the asymmetrical arrangement of the current electrodes.

Experimental procedure

The concentric pipettes and associated circulation assembly were rigidly fixed. Penetration of an alga was accomplished by the vertical movement of the chamber assembly. During this procedure both circulation systems were stopped. The cell was impaled with the tip of the inner pipette withdrawn into the bore of the outer capillary, but after entrance into the vacuole, the inner pipette was thrust downward so that it projected 2 mm below the bevelled tip of the outer pipette. The reason for this operation was that the inner, potential detecting pipette, in a recessed position, is strongly affected by the flow of current through the outer pipette. In its exposed position it detects the vacuole potential rather than a spurious potential drop across the resistance of the current carrying capillary.

An hour or so after penetration, when the cell maintained a constant P. D. of 80—90 mV, the vacuole perfusion was begun. This was initiated by opening the input stopcock while maintaining the output stopcock in the closed position. A hydrostatic

pressure was thus applied to the cell wall of the alga, and if an imperfect seal had been formed around the penetrating capillary, a sharp drop in the P. D. followed. With cells which withstood this test, the output stopcock was then opened and several ml of solution allowed to flow out at the disconnected air input T-tube. This operation eliminated sap and occasional gametes, lodged in the vacuole, from the circulating solution. Continuous circulation was then begun by connecting the air hose to the T-tube.

Influx and efflux determinations were made by adding labelled solutions (Na^{22}, Na^{24}, and Cl^{36}) to either vacuole or external solutions and making appropriate withdrawals for flux analysis while simultaneously recording the short-circuit current. Samples were transferred to aluminum planchettes, dried, and counted with an end window counter and automatic sample changer. Simultaneous influx and efflux measurements, utilizing a double label technique were not employed.

Most of the flux analysis were performed at room temperature (21—25° C). Some sodium fluxes were measured at 15° C and although the short-circuit current was smaller at this temperature, the flux percent of the current was the same as those measured at room temperature.

Protoplasm potential difference (P. D.)

Halicystis ovalis is turgid with a pressure of about 5 cm of water. The thickness of the protoplasmic layer, though small (10 μ), is large enough to accommodate an electrode with a tip diameter less than one micron. However, there is difficulty in penetrating the protoplasm due to the mechanical resistance of the outer cellulose wall. When an electrode is micro-manipulated toward a cell, a dimple appears at the point of contact. With penetration of a cell the dimple disappears and the electrode plunges through the protoplasm and into the vacuole. Two techniques were employed to overcome this difficulty. First: after penetration, a slight withdrawal of the electrode re-located the tip in the protoplasmic layer, but since the tip is tapered, the electrode in the withdrawn position allowed a low resistance leakage pathway and resulted in a variably shunted P. D. With time, however, the alga formed a seal around the electrode which eliminated the shunt pathway. Second: since the obstacle to penetration into the protoplasm was an initial yielding of the cellulose wall against the cells turgidity, an externally applied force, by means of a glass ring, was brought to bear on the cell which increased the pressure on the wall. Many penetrations were then accomplished with no dimpling and with the micro-electrode lodging in the protoplasm in the first thrust.

Results

Table I lists the concentrations of the major monovalent ions in sea-water and in the vacuole sap, the ion ratio in the two solutions, and the electrical potential difference across the protoplasm of *H. ovalis*. In the absence of a force due to solvent drag, an equation developed by Ussing (1949) and Teorell (1949)

$$M_1/M_2 = (a_2/a_1)\ \exp\ [((zF/RT)\ (E_m))] \qquad (1)$$

describes the flux ratio of a passively diffusing ion. The fluxes, M_1 and M_2, are the amounts of the ion diffusing through unit area of a membrane in unit time with the subscript denoting the direction of the diffusion; a_1 and a_2 are the activities of the ion in the respective solution, z, the valence, F, R, and T,

Table I. Concentrations in meq/l H_2O of the major monovalent ions in sea-water and vacuolar sap, after Brooks, (1929). The sign of the P.D. is the potential of the vacuole with reference to the environment at zero potential

	K	NA	Cl	P.D.
Sea-water	12.0	498	523	
Sap (H. ovalis)	337	257	543	−80 mV
Ratio (sap: sea-water)	28.1	0.52	1.04	

Concentrations in meq/l H_2O. The K and Na determinations made by Brooks (1929). The sign of the P.D. is the potential of the vacuole with reference to a grounded environment.

have their usual meaning, and E_m is the electrical potential difference across the membrane. Expressed in terms of electrical potential the equation has the form

$$(RT/F)\ ln\ M_1/M_2 = (RT/F)\ ln\ a_2/a_1 + E_m. \qquad (2)$$

In the quasi-equlibrium state of the normal resting cell, the left-hand member of this equation is zero for all ions that are not consumed in some metabolic reaction. Therefore, a rough test for a passive ion distribution is to insert the observed P. D. and the ion concentrations of the two solutions into equation (2), where the two right-hand members must cancel one another. In *H. ovalis*, the potassium ion fits this criteria perfectly, but both sodium and chloride are not in electro-chemical equilibrium in sea-water and vacuole sap. These two ions were therefore investigated since it seemed probable that they were actively transported.

The flux measurements were obtained from cells which had sea-water solutions inside and out and whose P. D. was short-circuited to zero. Under these conditions, equation (2) indicates that passively diffusing ions will experience no net flux and thus will contribute nothing to the short-circuit current. The influx and efflux determinations of each ion, Na and Cl, were performed in separate experiments and on different cells. The short-circuit current varied widely in different cells, but all the fluxes varied in a similar manner so that fluxes, determined on different cells, were compared on the basis of the flux percent of the short-circuit current.

Table II a lists the range and the mean of the sodium fluxes. The P. D. of the perfused cell before short-circuiting is tabulated in column 2. In column 5, the flux is expressed as a percentage of the short-circuit current. The mean efflux of sodium, as the percent of the short-circuit current was 48.7 %, S. D. = 5.2, while the mean sodium influx was 9.5 %, S. D. = 1.6. Therefore, a net active extrusion of sodium accounted for a mean 39.2 %, S. D. = 5.4, of the short-circuit current. Analysis of the chloride fluxes, Table II b, indicated a

Table II a and b. Sodium flux (a), chloride flux (b), and short-circuit current in cells with the sodium flux (a) or by the chloride flux (b)

Number of expts.		P.D. (mV)	Efflux pmol/cm²sec	Short-circuit μa/cm²	μa (flux) (×100)/ μa (short)
10	range	75—82	16—226	4—38	40—59
	mean	79	74	13	48.7, S.D. = 5.2
			Influx		
8	range	75—81	6—20	8—14	6—14
	mean	79	10	10	9.5, S.D. = 1.6
				mean net efflux = 39.2, S.D. = 5.4	

Sodium flux and short-circuit current in cells with sea-water inside and out.

net active influx of this ion which represented a mean 57.6 %, S. D. = 5.3, of the short-circuit current. The magnitude of the standard deviation indicates a variability in the flux percent values which will be discussed later.

Protoplasm P. D.

In the majority of penetrations, the protoplasm potential difference, detected by both techniques, was identical with the vacuole P. D. Therefore the total P. D. across the protoplasm of this alga develops at the outer membrane and there is no P. D. across the vacuole membrane.

Discussion

The behavior of the vacuole P. D. toward changes in ion concentrations in the sap and sea-water indicates the presence of two membranes in the protoplasm of *H. ovalis*; one at the cellulose wall interface and the other bordering the vacuole. Large transient changes in the P. D. result from variation of the potassium ion concentration in the external solution, whereas the P. D. is quite insensitive to similar changes in the vacuole solution (BLINKS 1932, 1935). If the observed P. D. depended only on the difference in chemical composition of sap and sea-water, replacing sap with sea-water in the vacuole of the cell should abolish the potential. This, of course, is not the case as the experiments presented in this paper indicate.

H. ovalis is therefore a three compartment system; sea-water, protoplasm and sap, with the outer and vacuole membranes serving as semi-permeable boundaries. The protoplasm has not been chemically analysed but it is probable that a large fraction of its anions are organic and impermeable; and due to the resulting Donnan equilibrium, the protoplasm would have a lower water activity than the solutions at its borders. Unless the membranes were unyielding and pressure resistant, the protoplasmic compartment would be osmotically unstable toward both sea-water and sap.

sea-water inside and out. Column five tabulates the percentage of short-circuit current carried by

Number of expts.		P.D. (mV)	Efflux pmol/cm²sec	Short-circuit μa/cm²	μa (flux) (×100)/ μa (short)
10	range	76—84	54—191	26—55	14—25
	mean	80	102	39	23.7, S.D. = 3.7
			Influx		
10	range	80—85	247—638	29—67	74—91
	mean	82	426	50	81.3, S.D. = 3.7
				mean net influx = 57.6, S.D. = 5.3	

Chloride flux and short-circuit current in cells with sea-water inside and out.

In the quasi-equilibrium state maintained by living cells, actively transported ions behave virtually as impermeable ions. In cells whose cytoplasm contains a relatively large concentration of impermeable anions, an osmotically stable system can be brought about by actively extruding an equivalent amount of either an anion or a cation. At equilibrium, the electrochemical potential of permeable ions is the same in the two compartments of such a double Donnan equilibrium and since the membrane P. D. is a function of the concentration gradients and relative membrane mobility of permeable ions, the magnitude of the P. D. depends on the sign of the extruded ion. When both the extruded and impermeable ions have the same sign and equivalent concentration, there is no passive ion concentration gradient and the P. D. of the system is zero; but when the two ions have different signs, a P. D. exists across the membrane.

In achieving osmotic stability, a function of the ion pumps observed in *H. ovalis* can be explained as the development of a double Donnan equilibrium between protoplasm and environmental sea-water, by the extrusion of sodium, and between protoplasm and the sap by the extrusion of chloride. The electrical potential of the protoplasm suggests that sodium and chloride are actively transported by the outer and vacuole membrane respectively. Support for this anatomic location of the pumps comes from experiments on the brackish water alga, *Nitellopsis obtusa*. By a compartmental analysis with radio-isotopes, MacRobbie and Dainty (1958) found that this cell actively extrudes sodium and actively accumulates chloride. They located the active sodium transport at the outer membrane and the chloride at the vacuole membrane.

Electrical equivalent circuit

The magnitude of the standard deviation of recorded flux percent of short-circuit current indicated a variability of this quantity which was greater than the estimated contribution of experimental error. This variability is thought

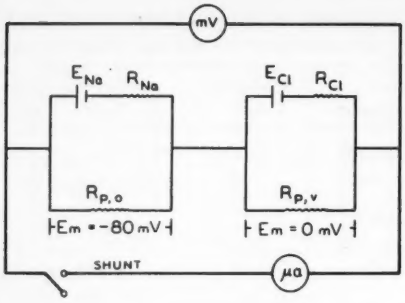

Fig. 2. A proposed electrical equivalent circuit of the membrane properties of *H. ovalis*. E_{Na} and C_{Cl} represent the electromotive equivalent of the sodium and chloride transport potentials. R_{Na} and R_{Cl} are the resistances of these ions in their active transporting mechanisms, $R_{p,o}$ and $R_{p,v}$ are the resistances to passively diffusing ions encountered at the outer and vacuolar membranes respectively. E_m indicates the membrane potential.

to result from the procedure of individual flux analysis. Since the short-circuit current is composed of at least two active fluxes, it is possible that the sum of these two quantities represents a constant fraction of this current, while individual components could vary, compensatingly, in their contribution. An analysis of this theory can be made by considering the forces acting on and the permeabilities of ions in terms of their electrical equivalents. Equation (2) is expanded

$$(RT/F)\ ln\ M_1/M_2 = (RT/F)\ ln\ a_2/a_1 + E_m + E_a \qquad (3)$$

to include a term, E_a, which is defined as an active transport potential (Ussing 1951, Linderholm 1952). But although E_a has the units of electromotive force, it can be evaluated only indirectly. Direct electrical measurements across an actively transporting membrane detects the membrane potential, E_m, and not E_a. However, E_m is a function of passive ion gradients and their permeability coefficients and the dynamic character of these gradients is maintained by active ion transports.

Fig. 2 is a diagram of the electrical properties of *H. ovalis* in an equivalent electrical circuit. Since the current, under short-circuit conditions, generated by the sodium and chloride transport systems, is in the same direction, the forces responsible for this current can be represented by two electromotive forces, E_{Na} and E_{Cl}, in series; E_{Na} at the outer membrane and E_{Cl} at the vacuole membrane. R_{Na} and R_{Cl} are the resistances encountered by these ions in their respective pumps. $R_{p,o}$ is the resistance of passively diffusing ions in the outer membrane and $R_{p,v}$ is the corresponding resistance at the vacuole membrane. Since the membrane potential, E_m, is a function of passive ion gradients, it has a finite value at the outer membrane but is zero at the vacuole membrane. When either of the resistances R_{Na} and R_{Cl} is varied, while maintaining other circuit equivalents constant, the fractional composition of the total current will be varied. If $R_{Na} >> R_{Cl}$, even when $E_{Na} = E_{Cl}$, flux measurements would indicate that the short-circuit current was composed almost

entirely of net chloride influx. Varying E_{Na} and E_{Cl} produces the same result but no change in the current composition accompanies changes in the passive resistances. Only the magnitude of the current is altered in this case.

Experimental results indicate that the electrical properties of *H. ovalis* are highly variable quantities. Many experimentally altered conditions, *i. e.*, decrease in oxygen tension, short-circuiting, metabolic poisons, ion substitution of the external solution, produce large increases in the total resistance of the cell. Even the cusp-shaped depolarisation response of this cell to increases in external potassium appears to be an increase in the membrane resistance, probably largely to the potassium ion, which decreases the original diffusion potential (BLINKS 1940).

The experimental proof of this theory will be found in the simultaneous influx and efflux measurements of both ions in one cell with a quadruple label technique.

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From the Department of Physiology, Karolinska Institutet, Stockholm, Sweden

A Study of Pressure-time Curves Obtained in the Occluded Renal Artery in Cats at Different Venous Pressures

By

ARNE ÅSTRÖM

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Abstract

ÅSTRÖM, A. *A study of pressure-time curves obtained in the occluded renal artery in cats at different venous pressures.* Acta physiol. scand. 1960. *49.* 10—20. — The magnitude of the minimum arterial pressure for flow in the kidney at different venous pressures (VP) has been studied in anesthetized cats by observing the pressure drop in the renal artery after interruption of its flow for 2.0—2.5 min. *At normal VP* flow became "practically zero" (<0.0067 ml per min) when the renal arterial pressure (RAP) was 12.5 ± 3.5 (S. D.) mm Hg and the mean arterio-venous (A—V) pressure difference 7.6 ± 3.0 mm Hg. The cessation of flow under these conditions has been attributed to the collapsing effect upon the vessels of tissue pressure. *At elevated VP* the RAP usually falls to the level of VP. However, in four of twelve cases an A—V pressure difference of 2—6 mm Hg was observed and has been attributed to a high vascular tone and/or high tissue pressure. This A—V pressure difference at elevated VP is absent in the denervated preparation. Measures increasing vascular tone tend to augment the difference. Elevation of VP for some minutes causes an increase in resistance to flow which will influence the time course of the pressure drop and, in the case of constant volume perfusion, the value of the minimum arterial pressure for flow is elevated for 4—5 min after restoration of VP to the normal level. This effect of VP elevation is qualitatively similar to that produced at normal VP by perfusion of fluids with a lowered colloid osmotic pressure. The results seem fully explicable without invoking a hypothesis of a "venous arteriolar reflex".

Recently, experimental data have been presented indicating that raising the venous pressure in an isolated vascular region produces an increase in resistance to flow in the same region. This effect has been attributed to an active vaso-

constriction elicited from the venous side by way of local reflex mechanism ("venous arteriolar reflex", GASKELL and BURTON 1953).

In the kidney vascular resistance has been shown to increase as a function of venous pressure. OCHWADT (1956) concluded that this effect of increased venous pressure, which could be abolished by KCN or Novocain, was a sign of active autoregulation which in turn seemed to be controlled by the pressure difference between the inside and the outside of the kidney vessels. HADDY (1956), on the other hand, attributed the change in resistance following elevation of the venous pressure to an active "venous arteriolar reflex" of principally the same kind as that described by BURTON and his collaborators in different peripheral vascular areas. Later HADDY *et al.* (1958) concluded that venous pressure elevation in the kidney increased resistance by active vasoconstriction as vell as by "passive vasoconstriction". The "passive vasoconstriction" was thought to be due to a reduction in the transmural pressure as elevation of venous pressure produced an increase in the interstitial pressure.

The present investigation was undertaken to study whether additional information, regarding the mechanism by which kidney vascular resistance increases with venous pressure, could be obtained from the analysis of pressure-time curves as has previously been done by GIRLING (1952) and others in their study of the "venous arteriolar" reflex in different peripheral vascular areas.

Methods

Twenty-nine cats (2.0—2.5 kg) were anesthetized with chloralose (50—60 mg per kg) or chloralose-urethane (40 plus 100 mg per kg) after ether induction. The left kidney was exposed by a mid-line incision. Its vessels were prepared by blunt dissection with special care to minimize damaging the nerves and all small vessel branches to the perirenal fat tissue carefully ligated up to the hilus. The vessels were cannulated and connected with the femoral vessels on the right side. Pressures were photographically recorded by rubber membrane manometers or strain gauge transducers. The measuring and recording apparatus has been previously described in detail (ÅSTRÖM and YAMADA 1958).

Pressure-time curves were obtained by occluding the arterial blood supply to a rubber bag inside a water filled capsule for 1.5—2.5 min. Following occlusion the renal arterial pressure, as recorded from the capsule, fell at first sharply and then levelled off in an asymptotic relationship to a horizontal plateau. During the occlusion the renal arterial pressure was read each half minute from a one-arm, small bore mercury manometer or from a strain gauge pressure transducer electromanometer (Elema, Sweden) in parallel with the recording rubber membrane manometer.

During the pressure-time curve determinations at elevated venous pressure, the venous outflow was collected in a reservoir. The final pressures are called P_{Af} and P_{Vf} (*f*inal *a*rterial or *v*enous *p*ressure), if the arterial flow as calculated from the slope of the terminal part of the pressure-time curve was less than 0.0067 ml per min.

In some experiments the kidneys were removed and placed in a plastic container at a temperature of 38° C. Otherwise the experimental arrangements in these cases were the same as in the other experiments. In another series of six experiments intermittent

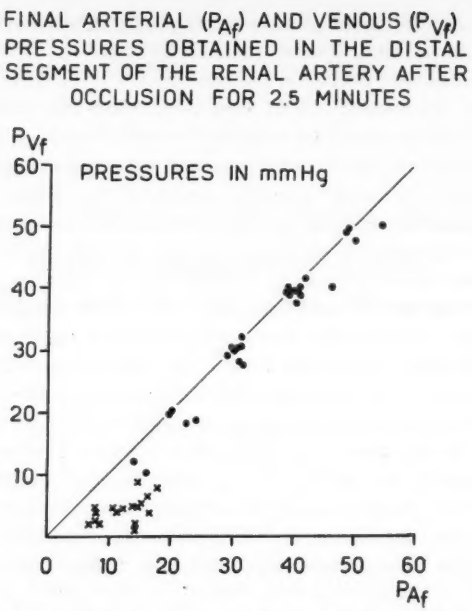

Fig. 1. Plot showing the relationships between P_{Af}'s and P_{Vf}'s obtained after 2.0—2.5 min occlusion.
× = values obtained at normal venous pressure (free drainage);
● = venous pressure set by the reservoir (12 animals).

local perfusion with dextran solutions was made with an adjustable flow rate pump model T-8, (Sigmamotor, Inc. Middleport, N. Y.). A "Windkessel" was used distal to the pump and all connecting Tygon tubings were warmed by temperature controlled air in polyethylene sleeves. An anticoagulant (heparin) was given every hour and all the perfusion solutions were maintained at 38° C during the experiments. The dextran solutions and heparin were kindly supplied by AB Pharmacia and Vitrum AB respectively.

Results

1. *Observations at normal venous pressure.* In 17 animals pressure-time curves were first obtained at normal venous pressure with free drainage of the venous outflow to the femoral vein. Within the occlusion time of 2.0—2.5 min the renal arterial pressure fell to approximately a horizontal level in the curve *i. e.* the arterial flow became "practically zero" (less than 0.0067 ml per min). At this point the arterial pressure (P_{A_f}) had an average value of 12.5 ± 3.5 (S. D.) mm Hg and the venous pressure at the same instant (P_{V_f}) was 4.4 ± 2.1 mm Hg. The P_{A_f} in all experiments was higher than the corresponding P_{V_f} and the

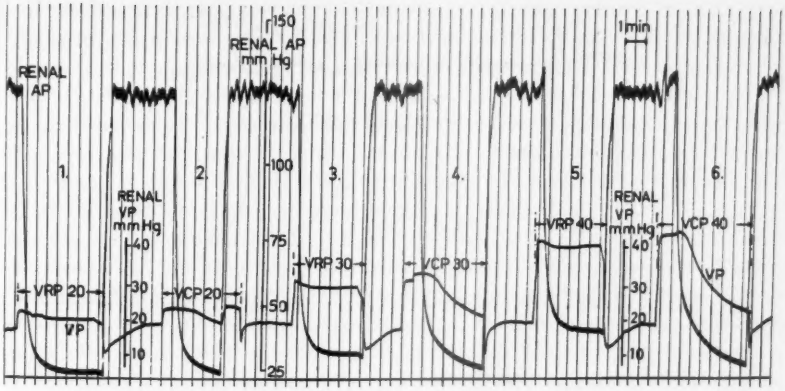

Fig. 2. Pressure-time curves obtained with two different ways of elevating venous pressure by venous reservoir (VR), as in tests 1, 3 and 5, or by partial obstruction of the venous return by a capsule valve (VC), as in tests 2, 4 and 6.
Note: Venous pressure could not be maintained during the occlusion, when the capsule arrangement was used.

mean $P_{A_f} - Pv_f$ difference was 7.6 $\pm$ 3.0 mm Hg as calculated from each corresponding pair of estimations. The results are given in Fig. 1 (values indicated by $\times$).

2. *Observations at elevated venous pressure.* In 12 animals the pressure-time curves were studied at different levels of elevated venous pressure. The maintenance of elevated venous pressure by partial obstruction of the venous outflow did not prove satisfactory. This was seen in a preliminary series of experiments in which on the venous side, in accordance with GIRLING (1952) and ROSENBERG (1956), a capsule arrangement similar to that on the arterial side was used. The typical differences in results obtained by this method in comparison with the results obtained when using a venous reservoir is illustrated in Fig. 2. In the tests in which a venous capsule was used (tests 2, 4, 6,) the venous pressure (VP) fell continuously during the occlusion period and this in turn prevented the arterial pressure from levelling out to a horizontal plateau. In these cases arterial pressure values at the end of comparable occlusion periods corresponded to different arterial flows (all more than 0.0067 ml per min) and this made comparisons difficult. In contrast, when the reservoir was used, the venous pressure remained practically unchanged during the occlusion even at the highest venous pressures (tests 1, 3, 5, Fig. 2). Only the reservoir method was therefore used in the remainder of the study.

With the reservoir method a retrograde flow (usually about 2—4 ml at venous pressures of 30—40 mm Hg) was observed during the occlusion periods. The retrograde flow seem to be due largely to gradual filling of the renal veins but in part also due to filtration into the tissue spaces. The occurrence of this retro-

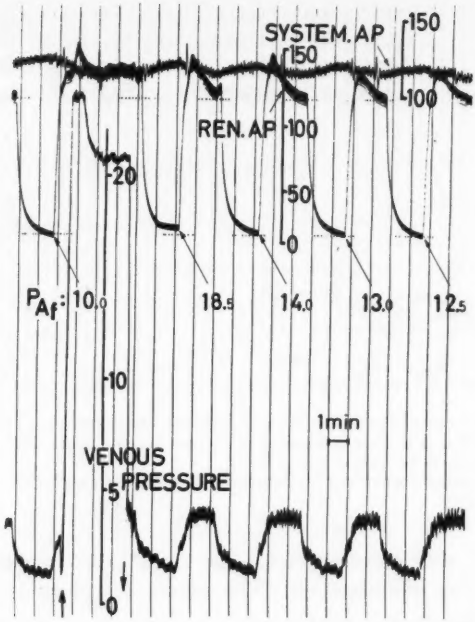

Fig. 3. After the first pressure-time curve venous pressure is elevated to 21 mm Hg for 3 min. The next pressure-time curve shows an elevated P_{A_f} (8.5 mm Hg above control) while the subsequent P_{A_f}'s gradually return to control level. Elevation of venous pressure also increased resistance to flow which is evident from the renal arterial pressure recording in between the occlusions. Sigmamotor pump set at 30 ml per min.

grade flow would seem to account for the failure to maintain venous pressure during the occlusion, when the capsule method was used.

In the series in which the pressure-time curves were determined at venous pressure of 20 mm Hg or more the P_{A_f} and P_{V_f} became identical in eight of the twelve cases. The eight experiments included two isolated kidneys. In four preparations the P_{A_f} was 2—6 mm Hg higher than the P_{V_f} at the end of the 2.0—2.5 min occlusion. The P_{A_f} observed at elevated venous pressure was never higher than the sum of the original P_{A_f} at normal venous pressure plus the applied venous pressure. The results are given in Fig. 1 (values indicated by •). At each level of venous pressure tested only one result (the first) from each animal is included. In half of the cases the kidneys were perfused with blood at static pressures (arterio-venous pressure difference 90 mm Hg) for a short time before the arterial flow was occluded. However, small differences in renal arterial pressure at the beginning of the occlusion influenced only the initial rate of fall in pressure and not the terminal part of the pressure-time curve (P_{A_f}).

3. *Resistance of flow and P_{A_f} after release of elevated venous pressure.* In the experiments in which tests were performed at elevated venous pressure it was often observed that the P_{A_f} after normal venous pressure had been reestablished, was often higher than the control before the elevation of the venous pressure. In such cases, 2—3 min after the period of elevated venous pressure, blood flow was also decreased; *i. e.* the initial part of the curves showed a slower rate of fall in pressure. This effect of venous pressure was most regularly produced in experiments with constant flow perfusion with blood as well as dextran solution in the manner illustrated in Fig. 3. A series of pressure-time curves are performed, one before and four after elevation of venous pressure to about 21 mm Hg for 3 min by partial occlusion of the venous return. The renal arterial pressures were *read* from the electromanometer set at high sensitivity in the low range of pressure and the pressure-time curves *recorded* with a rather insensitive optical membrane manometer. 2.5 min after the restoration of the venous pressure to the original level the P_{A_f} is elevated from the previous control value of 10.0 to 18.5 mm Hg. The successive four pressure-time curves show that the P_{A_f} gradually returns to the original level. The renal arterial pressure in between the occlusions also gradually returns to the control level indicating that the resistance to flow is gradually being normalized. The pressure-time curves were obtained by switching off the sigmamotor pump which was set at a flow rate of 30 ml per min. The results were qualitatively identical in the four experiments in which this effect of elevated VP was particularly studied by the constant flow technique.

4. *Role of vascular tone.* To study the possible role of vascular tone for the $P_{A_f} - P_{V_f}$ difference sometimes observed at elevated venous pressure, vascular tone was increased by electrical stimulation of renal nerves by a Grass model S4E stimulator (frequency 1—20 per sec) and infusion of noradrenaline into the renal artery. The changes produced in the pressure-time curves during blood perfusion by these procedures were, however, such that a final horizontal plateau of pressure was usually not obtained. The difficulty in altering P_{A_f} by measures increasing the vasomotor tone have been dicussed earlier for the venous pressure range below 10 mm Hg (YAMADA and ÅSTRÖM 1959).

In experiments in which perfusions of the kidney in situ were performed with dextran an increase in the $P_{A_f} - P_{V_f}$ difference could, however, be produced by adding noradrenaline to the perfusing fluid. Fig. 4 shows a typical response. Here dextran perfusion with the sigmamotor pump was started as marked by an asterisk following which venous pressure was elevated to about 22 mm Hg. Fig. 4 A is obtained with ordinary dextran solution while in B, noradrenaline was added to the perfusing dextran (1 μg per 25 ml). The first pressure-time curve (A) shows a difference between the final arterial and venous pressures of 2 mm Hg. The pressure-time curve in B, started when the noradrenaline induced renal arterial pressure was 160 mm Hg, yielded a $P_{A_f} - P_{V_f}$ difference of 4 mm Hg after the 1.5 min stoppage of the pump. The flow was set at 15 ml

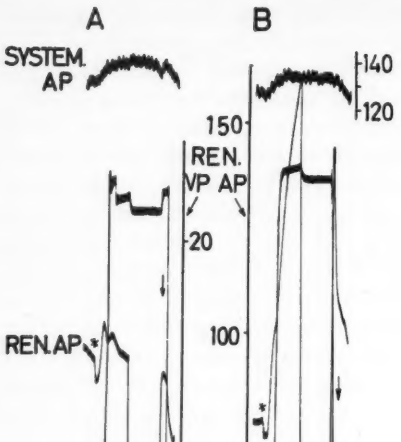

Fig. 4. Pressure-time curves obtained during perfusion with dextran (A) and noradrenaline-dextran (B). Increasing vasomotor tone in B augments the P_{A_f} — P_{V_f} difference.
* = start of perfusion by pump set at 15 ml per min.

per min during the dextran perfusions and between the tests the kidney was allowed to recover for 10 min on normal circulation with blood.

It is noteworthy that in spite of the considerable increase in renal vascular resistance during noradrenaline-dextran perfusion, as indicated by the 85—90 mm Hg increase in renal arterial pressure, the elevation of P_{A_f} is quite small (2 mm Hg). This could, of course, be due to some extent, to a local distruction of noradrenaline in the kidney while circulation is stopped for 1.5 min. However, the most important factor seems to be that considerable changes in resistance to flow at normal rates will be accompanied by only minor changes in P_{A_f} as has been discussed previously (YAMADA and ÅSTRÖM 1959).

The influence of lowering vascular tone on the P_{A_f} — P_{V_f} difference is more difficult to study by the use of this pressure-time curve technique. First of all, the difference showed a tendency to disappear spontaneously with time, therefore to decide with certainty whether the reduction of the P_{A_f} — P_{V_f} difference observed was due to a deliberately performed procedure or not was

difficult. A $P_{A_f} - P_{V_f}$ difference was most commonly seen at the beginning of an experiment. It has not been observed at venous pressures of 15 mm Hg or more in denervated or isolated kidneys (four and two cases respectively).

5. *Role of colloid osmotic pressure of the perfusing fluid.* The results obtained after elevation for some time of venous pressure seemed to suggest that increased tissue pressure (intrarenal pressure) might be a factor influencing the resistance to flow in this type of experiments. To investigate this possibility further a series of experiments were performed in which the colloid osmotic pressure of the fluid perfusing the kidney was varied.

Perfusion with Ringer solution instead of blood is known to increase the resistance to flow in the kidney after a very short time (WHITTAKER and WINTON 1933). When in the present investigation normal circulation with blood was resumed after infusion of a few ml of Ringer solution for 2—3 min and then a pressure-time curve obtained, the rate of fall in pressure was markedly decreased and within the usual occlusion time "practically zero" flow was usually not obtained. The changes were qualitatively the same as those seen in the pressure-time curves obtained when noradrenaline was administered intra-arterially or when electrical stimulation of renal nerves was performed in the blood perfused preparation.

In another type of experiment dextran solutions at 38°C were perfused by a sigmamotor pump and pressure-time curves obtained by stopping the pump for two minutes. Dextran solutions whose colloid osmotic pressure was somewhat higher than that of blood yielded a pressure-time curve in which the rate of fall in pressure was rapid and a final horizontal part was readily obtained. In contrast to this, the same dextran solutions diluted to double volume with 0.9 per cent saline gave a pressure-time curve with a more gradual rate of fall in pressure and a P_{A_f} value usually some 2—6 mm Hg higher than with non-diluted dextran solution. Thus the difference in osmotic pressure caused the same changes as observed with blood before and after some time of elevated venous pressure (Fig. 3).

Discussion

It has been shown that at venous pressures below 10 mm Hg the pressure in the distal segment of the occluded renal artery after 2.0—2.5 min falls, but is always greater than the venous pressure. The minimum arterio-venous pressure difference necessary to maintain perceptible flow (more than 0.0067 ml per min) has a mean value of 7.6 $\pm$ 3.0 (S. D.) mm Hg (17 cases). The minimum arterial pressure for flow (P_{A_f}, in this situation corresponding to the "critical closing pressure") in this range of venous pressure was 12.5 $\pm$ 3.5 mm Hg which is of the same order of magnitude as the average "yield pressure" of 10.7 mm Hg in the kidney of the dog (RITTER 1952). It also agrees with the value (10—20 mm Hg) found for the intercept on the pressure axis of

pressure-flow curves in the kidney of the dog (WHITTAKER and WINTON 1933, SELKURT 1946).

The minimum arterio-venous pressure difference required for flow found in this study is practically identical with that previously reported from this laboratory (YAMADA and ÅSTRÖM 1959). In this previous study it was concluded that the factors determining the P_{A_f} in the normal range of venous pressure were largely vasomotor tone and tissue pressure. Since in the kidney tissue pressure is higher than in most other vascular areas (10 mm Hg given by WINTON (1953) and by GOTTSCHALK (1952)), it was also concluded that in this organ the tissue pressure under the experimental conditions used is relatively more important than vasomotor tone. Thus, in the kidney the tissue pressure seems to be mainly responsible for the reduction in the caliber of the resistance vessels to such a degree at an intraluminal pressure of about 13 mm Hg (as found here) that flow ceases while there still remains an arterio-venous pressure difference of 8 mm Hg (average in the present investigation). This conclusion indicates that most kidneys under the experimental conditions here had a negligible active vasomotor tone. It also follows that a "critical closure" (BURTON 1951) of the kidney resistance vessels need not be assumed and that the P_{A_f} more appropriately should be considered as a "yield pressure". Absence of the phenomenon of "critical closure" in the dog kidney has recently been reported by HINSHAW, DAY and CARLSON (1959).

At elevated levels of venous pressure (15—60 mm Hg) the arterial and venous pressures at the end of an occlusion period usually become identical in the innervated kidney and regularly so in the denervated preparation. In four of twelve innervated preparations in this study a $P_{A_f} - P_{V_f}$ difference of 2—6 mm Hg was observed, with an arterial flow of "practically zero" (less than 0.0067 ml per min) following occlusion for 2.0—2.5 min. The P_{A_f} at elevated venous pressure was never greater than the sum of the control P_{A_f} (determined at normal venous pressure) and the applied venous pressure. Increased venous pressure has been found to elevate the intrarenal pressure (GOTTSCHALK, 1952). WAUGH and HAMILTON (1958) in the oil-perfused kidney found that renal resistance remained constant with equally raised arterial and venous pressures and concluded that a generalized distension of the intrarenal vasculature was prevented by a compensating rise in extravascular pressure equal to the increment in venous pressure.

In the oil-perfused kidney engorgement of the highly distensible venous system located inside the rigid kidney seemed to be the cause of the rise in the intrarenal pressure. Increasing the venous pressure when blood or dextran perfusion is used would be expected to increase filtration from the capillaries so that in these cases an elevated intrarenal pressure, produced at elevated venous pressure, would be expected in part to remain for some time after lowering of the venous pressure. However, in the case of perfusion with oil, which is claimed not to penetrate the capillary pores, the intrarenal pressure

is immediately reversible by lowering the venous pressure (WAUGH and HAMILTON 1958). Evidence for a persisting increase in resistance to flow in the kidney after some minutes elevation of venous pressure to about 20 mm Hg has been found in this investigation both with blood and dextran perfusion (Fig. 3). The effects on the pressure-time curves after elevation of venous pressure has also been found to be qualitatively similar to those obtained by lowering the colloid osmotic pressure of the perfusing fluid. It would seem likely that the common cause of the persisting increase in vascular resistance is an increase in intrarenal pressure.

When pressure-time curves are obtained at elevated venous pressure, the arterial pressure at the end of an occlusion period cannot fall below the value of the applied venous pressure. If the resistance vessels are distensible this increased intravascular pressure would leave these vessels with a larger caliber at the end of the occlusion and due to the decreased resistance to flow an equalization of the arterial and venous pressure would be expected at elevated venous pressure. If the distension of the resistance vessels is largely prevented by an increase in interstitial pressure, equal to the increment in venous pressure according to WAUGH and HAMILTON (1958) an equalization of the arterial and venous pressures would still be expected in the majority of cases, since the intrarenal pressure in this case is assumed to be equal to the venous pressure, while at normal venous pressure it is some 8—10 Hg higher than the venous pressure. An equalization of the arterial pressure to the level of the venous pressure, if the latter were raised to 20 mm Hg or more, was observed in eight of the twelve cases in this investigation.

Since the distension of the resistance vessels in the kidney at high intravascular pressure, produced by elevating venous pressure, seems to be counteracted by the concomitant rise in intrarenal pressure, a $P_{A_f} - P_{V_f}$ difference at the end of an occlusion period would be expected in preparations with an initially high resistance to flow. Such high resistance to flow at normal venous pressure could be due to high intrarenal pressure of high active vasomotor tone. Experimental evidence for high vascular tone as a factor determining the magnitude of the $P_{A_f} - P_{V_f}$ difference at elevated venous pressure has been presented above (Fig. 4).

In view of these findings the most likely cause for the $P_{A_f} - P_{V_f}$ difference, observed in four of twelve cases at elevated venous pressure, would seem to be a prevailing high vasomotor tone, alone or in combination with an initially high intrarenal pressure. If this interpretation be correct it would seem to be little reason to consider the P_{A_f} at elevated venous pressure as a "critical closing pressure". Also to explain the fact that its magnitude sometimes exceeds that of venous pressure (P_{A_f}) a reflex mechanism ("venous arteriolar reflex") need not be postulated. By blocking or section of the renal nerves, vasomotor tone is lowered and this seems to be sufficient to explain that a $P_{A_f} - P_{V_f}$ difference does not persist after denervation. Our observations also indicate

that the effect of elevated venous pressure upon vascular resistance in the kidney at normal flow rates is dependent on the prevailing vascular tone. These relationships will be further discussed in a subsequent paper.

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From the Department of Physiology, Karolinska Institutet, Stockholm, Sweden

Resynthesis of Adrenaline in the Rabbit's Adrenal Medulla During Insulin-Induced Hypoglycemia

By

S. Bygdeman, U. S. v. Euler and B. Hökfelt

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Abstract

Bygdeman, S., U. S. v. Euler and B. Hökfelt. *Resynthesis of adrenaline in the rabbit's adrenal medulla during insulin-induced hypoglycemia.* Acta physiol. scand. 1960. *49.* 21—28. — Resynthesis of suprarenal adrenaline in rabbits during insulin hypoglycemia was calculated on the basis of the amount of adrenaline present in the gland and the amounts produced, estimated from the urinary excretion of adrenaline during a given period. It was found that depletion of adrenaline in the gland of the insulin treated animal was accompanied by a resynthesis, amounting to more than twice the average normal content in 24 hours.

Increased secretion of adrenaline from the suprarenal medulla during the hypoglycemic phase after insulin was first observed by Houssay, Lewis and Molinelli (1924) and Cannon, McIver and Bliss (1924) and has since been amply confirmed directly or indirectly (Euler and Luft 1952, Dunér 1954, Goldfien *et al.* 1958). In a study of the effect of insulin on the adrenal medulla, Hökfelt (1951) found a pronounced reduction of the adrenaline content in the suprarenal glands of rats and rabbits after insulin injections. The lowest values were observed after 14 hours and the normal content regained only after some 6 days. Similar results were obtained by Udenfriend *et al.* (1953) who found that about half of the adrenaline which had disappeared from the adrenal glands after insulin administration in rats and in rabbits was restored in 72 hours.

Using smaller doses of insulin, West (1951) found a moderate depletion of adrenaline in the suprarenal glands of the rabbit with the lowest level

about 4 hours after injection. By the sixth hour the values were normal again, and after 8—12 hours a further increase was found, indicating a fairly rapid resynthesis. He found no discharge after severing the splanchnic nerves, or after injection of insulin into the suprarenal artery, indicating that insulin did not affect the suprarenal glands directly but only via the splanchnic nerve. The results of DUNÉR (1953), who found that a local elevation of the blood sugar level in the brain of the cat, with its head separately perfused, was followed by a decreased secretion from the suprarenal medulla, also indicate that the release is mediated by the secretory nerves.

The discharge of adrenaline and noradrenaline produced by direct electrical stimulation of the splanchnic nerve is, however, in many cases followed by a rapid resynthesis (HÖKFELT and MCLEAN 1950, HOLLAND and SCHÜMANN 1956, BYGDEMAN and EULER 1958). Different results were obtained by EADE and WOOD (1958) who did not find rapid resynthesis after splanchnic stimulation.

The slow repletion of adrenaline in the adrenal medulla after insulin is at variance with the relatively rapid resynthesis reported after splanchnic stimulation. One might therefore consider the possibility that insulin inhibits the synthesizing ability of the chromaffin cells in a more direct way, independently of innervation. To test this possibility we have denervated one gland in rabbits and determined the adrenaline content in the normal and the denervated gland separately after insulin. Any direct effect of insulin on the gland might then be detected, especially if the gland was made to secrete more intensely as with nicotine. Since these results were negative, further experiments were made in which adrenaline release during insulin hypoglycemia was estimated by measuring the adrenaline excretion in urine and the depletion in the glands. This estimation depends on the quantitative correlation between the amounts excreted in urine and the amount administered by intravenous infusion. Since the percentage of adrenaline excreted might vary in relation to the rate of infusion, it seemed necessary to measure the excretion for a series of different adrenaline infusion rates and plot the relationship on a curve. From the actually observed excretion rates in the insulin experiments the corresponding amounts released from the suprarenal medulla could then be read from the curve, assuming the same relationships between the amounts excreted and those secreted into the suprarenal veins as after intravenous infusions.

Methods

Insulin and nicotine in denervation experiments

Rabbits weighing 2.3—3.3 kg were used throughout. In one group of animals the left adrenal gland was denervated by section of the left splanchnic nerve below the diaphragm under nembutal anesthesia (40 mg/kg i.p.). One week later the animals were given regular, glucagon-free insulin (Vitrum) subcutaneously, having fasted for 15 hours. The insulin was given twice a day, 1 unit/kg, at the beginning of the experiment,

Table I. Content of adrenaline and noradrenaline in the suprarenal glands after injections of insulin. Left gland denervated. Expt. no. V and VI controls, the animals receiving 0.9 % NaCl instead of insulin

Expt. no.	Insulin dose in IU/kg	Duration of stimulation in hours	Denervated gland µg/kg b. wt.		Intact gland µg/kg b. wt.	
			adr.	noradr.	adr.	noradr.
I	1 + 1	6,9	27	0	3,4	0,2
II	1 + 1	7,25	22	0	1,6	0,1
III	1 + 2	9,1	31	0	6,9	0
IV	1 + 2	9,5	24	0	2,9	0
V[2]	—	8—9	41	0	49	0
VI[2]	—	8—9	32	0	37	0
VII[1]	1 + 2	7	24	0	14	0
VIII[1]	1 + 2	7	38	0	16	0
IX[1]	1 + 2	7	37	0	4	0
X[1]	1 + 2	7	31	0	13	0
XI[1]	—	7	43	0	41	0
XII[1]	—	7	36	0	35	0

[1] Received nicotine, 7 × 1 mg i. v.
[2] Controls, receiving no insulin.

and 1—2 units 4—5 hours later in order to maintain a low blood sugar level during the entire experiment. The blood sugar level was measured several times during the experiment by the method described by HAGEDORN, HALSTRÖM and JENSEN (1935). Six rabbits were given 9—10 injections of nicotine tartrate 1 mg/kg intravenously over a period of about 7 hours. Four of these received injections of insulin according to the scheme described above. Finally the animals were killed by air injection i. v. and the suprarenal glands removed. Each gland was extracted in 10 ml 5 % trichloracetic acid and the catechol amine content determined fluorimetrically according to EULER and LISHAJKO (1959).

Adrenaline infusion experiments

Rabbits anesthetized with 40 mg/kg nembutal i.p. were given i. v. infusions of known amounts of adrenaline by means of a slow injection device and the urine collected. The adrenaline solution was made by diluting a stock solution with saline, acidified to pH 3—4 with hydrochloric acid. An infusion rate of 10 ml per hour was used in all experiments. The concentration of the solution was adjusted to correspond to this infusion rate.

Anesthesia was maintained during the experiment by small additional injections of nembutal as indicated by changes in respiration and the corneal reflex. The adrenaline infusions were made in the femoral vein and the urine collected continuously with a catheter in the bladder. The urine was analysed for adrenaline and noradrenaline according to EULER and LISHAJKO (1959).

Insulin experiments with urinary adrenaline measurements

The animals were kept in individual cages and anesthetized with nembutal 40 mg/kg i.p. and the urinary bladder emptied by catheterization. Insulin was given

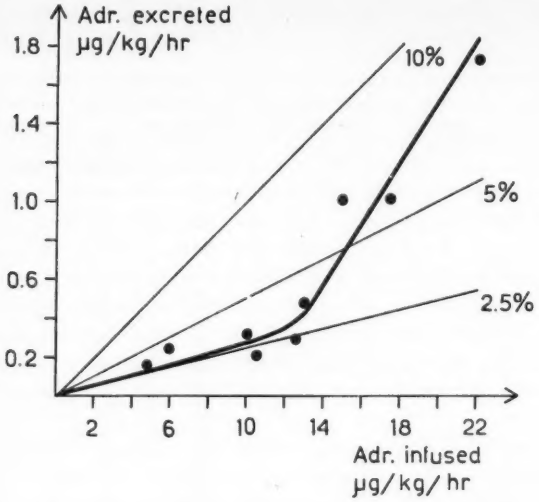

Fig. 1. Correlation between infused concentrations of adrenaline and simultaneously excreted amounts.

s.c. and the spontaneously voided urine collected with a funnel beneath the cage and let into a flask with N HCl giving a final pH of 2—3. The urine was allowed to flow between plastic rods which formed the bottom of the cage. The urine was then analysed for adrenaline and noradrenaline. The usual experimental period was 6—7 hours, but in one case the rabbit was kept in the cage for 72 hours during which time repeated injections of insulin were given. This animal received fresh grass during the nights. At the end of the experiment the urinary bladder was emptied either by pressure on the abdomen or with a catheter as described above. The animals were then sacrificed and the urinary bladder opened for recovery of residual urine. It never contained more than 0.5 ml urine. The suprarenal glands were removed and analysed as described above.

Results

I. *Insulin and nicotine after unilateral adrenal denervation*

Four animals were given insulin one week after the left adrenal gland had been denervated. The content of adrenaline and noradrenaline in the adrenal glands is shown in Table I. Only small amounts of adrenaline could be found in the intact gland as described by previous authors, but in the denervated gland the content was normal or only slightly decreased, compared with the control values. In two of the intact glands traces of noradrenaline were found, confirming previous findings (HÖKFELT and MCLEAN 1950).

Table II. Adrenaline secretion from the suprarenal gland of the rabbit during insulin stimulation

Expt. no.	Insulin dose in IU/kg	Duration of stimulation in hours	Adr. content in stimulated gland μg/kg/gland	Loss of adr. μg/kg/gland	Adr. excretion in urine μg/kg/hour	Calculated secretion of adr. μg/kg/gland		Resynthesized adr. μg/kg/gland	
						total	per hr	total	per hr
XXIV	1 + 2	6.7	0[1]	43[1]	0.58	47	7	4[2]	0.6[2]
XXV	1 + 2	10.5	0[1]	43[1]	0.39	65	6.2	22[2]	2.1[2]
XXVI									
1st day....	2 + 1				0.12				
1st night ..					0.074				
2nd day ..	2 + 1				0.75				
2nd night..		72	19	24	0.12	197	2.7	173	2.4
3rd day ..	2 + 3 + 3				0.46				
3rd night..					0.12				
XXVII	1 + 2	6.1	30	13	0.39	38	6.2	25	4.1
XXVIII....	2 + 2	6.4	4	39	1.25	59	9.2	20	3.3
XXIX.....	2 + 2	6.6	11	32	0.79	51	7.7	19	2.9
Controls	—	—	—	0	0.04	—	< 1.0	—	< 1.0

[1] Adrenaline content not determined. Normal content taken as 43 μg/kg/gland; after insulin, gland assumed to be emptied.

[2] Minimal values.

In the nicotine experiments no consistent change in the adrenaline content was observed. When insulin was given, the content was decreased as usual in the innervated gland but not in the denervated one (Table I).

The blood sugar was maintained at a level of 40—60 mg % throughout the shorter experiments. Repeated doses of glucose had to be given to counteract convulsions.

II. *Infusion experiments*

The amounts of urinary adrenaline excreted during different infusion doses of adrenaline are given in Fig. 1. The value for normal adrenaline excretion, 0.04 μg per kg per hour has been subtracted from the total output. The rate of infusion varied from 4.8 to 22 μg/kg/hr in 9 experiments and the infusion time was 3—6 hours. As seen in the figure the excretion increases with increasing dose. The curve drawn in the figure has been used in order to calculate the secretion from the adrenal glands in the following experiments from the excretion in the urine.

III. *Insulin experiments*

From Table II it can be seen that the amounts of adrenaline released during insulin hypoglycemia, as calculated from the excretion figures, are considerably in excess of the deficit in the gland, indicating varying but relatively high rates of resynthesis. The depletion shows varying degrees in the animal exposed to insulin hypoglycemia. Thus in the case of hypoglycemia for 72 hours the content was relatively high, about half the normal value.

To calculate the amounts of adrenaline released it has been necessary to use a figure for the normal content of the gland. In seven controls the average adrenaline content was found to be 43 μg/kg/gland. The same figure has been reported by KRONEBERG and SCHÜMANN (1959).

In rabbits XXIV and XXV the glands were not analysed at the end of the insulin stimulation experiment. Assuming that depletion was complete the resynthesis represents a minimum figure.

If the mean rate of resynthesis is calculated in experiments XXIV—XXV it varies between 0.6—2.1 μg/kg/hr/gland and the mean actual secretion varies between 6.2—7.0 μg/kg/hr/gland. In experiments XXVII—XXIX the corresponding values are 2.9—4.1 and 6.2—9.2 μg/kg/hr/gland.

As stated above the resynthesis figures are probably too low in the first two experiments. In experiment XXVI no insulin injections were given during the nights which may account for the lower figures for resynthesis and secretion, 2.1 and 2.4 μg/kg/hr/gland respectively. The excretions at night were lower than during the day but higher than during normal resting conditions as seen in the table.

Discussion

The results presented in Table I are in good accordance with those of HÖKFELT (1951) and UDENFRIEND *et al.* (1953) who found a strong depletion of adrenaline in the innervated suprarenal after administration with insulin. The near normal content of adrenaline in the denervated gland shows clearly that the insulin effect is mediated via the splanchnic nerve. A rapid rebuilding as found by WEST (1951) has not been observed, probably because a prolonged insulin action was attempted in our experiments, using two doses.

PITKÄNEN (1956) observed that moderate doses of insulin (0.2 unit per rat) caused a large increase in the adrenaline excretion. The response to insulin injections, repeated at 2-day intervals was temporarily decreased but returned to the original high values in 4—6 days. The adrenaline content in the suprarenals was not estimated, however, in these experiments.

SOURKES, DRUJAN and CURTIS (1959) also observed increased excretion of adrenaline in urine over a period of 5 days in patients receiving a daily injection of 0.2—1 i. u. per kg of insulin.

The experiments presented in Table II of this paper indicate that the gland is still capable of resynthesising adrenaline during insulin hypoglycemia. Why under such circumstances it takes about 6 days to regain the normal content of adrenaline in the rat and the rabbit adrenal medulla as found by HÖKFELT (1951) and UDENFRIEND *et al.* (1953) is still not understood. UDENFRIEND and WYNGAARDEN (1956), remarking on the slow turnover of catechol amines which they found in the suprarenal glands of insulin-treated rabbits also offered the alternative explanation that the synthesis takes place at a rapid rate but that most of the newly synthesized material is continually secreted into the blood, only small amounts being stored in the gland. The present experiments seem to support this idea.

The highest calculated amount of adrenaline released from one suprarenal gland after insulin stimulation, 153 ng/kg/min, is in good agreement with the rate of secretion reported for dogs by GOLDFIEN *et al.* (1958) and by SCHAEPDRYVER (1959) who found values reaching 195 and 88 ng/kg/min respectively. In DUNÉR's (1954) experiments on cats the corresponding figure was 120 ng/kg/min.

As the effect of insulin on the adrenaline release is mediated via the splanchnic nerve, the rapid resynthesis found is also in good agreement with the results obtained earlier by HÖKFELT and MCLEAN (1950) and HOLLAND and SCHÜMANN (1956) as well as with those of BYGDEMAN and EULER (1958) using electrical stimulation of the splanchnic nerve. Consequently it seems probable that a rapid resynthesis in the suprarenal gland should occur generally in such cases where the release is mediated via the splanchnic nerve. This is in contrast with the results obtained with acetylcholine (BUTTERWORTH and MANN 1957) or nicotine (BYGDEMAN and EULER 1958), suggesting that these drugs in some way alter the synthesising capacity of the gland.

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Acta physiol. scand. 1960. 49. 29—34

From the Departments of Physiology (1) and Pathology (2), Kungl. Veterinärhögskolan, Stockholm 51, Sweden

The Effects of Obesity, Pyometra and Diabetes Mellitus on the Fat and Cholesterol Contents of Liver and Spleen in the Dog

By

J. S. FINLAYSON[1] (1), L. KROOK[2] (2) and S. LARSSON (1)

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Abstract

FINLAYSON, J. S., L. KROOK and S. LARSSON. *The effects of obesity, pyometra and diabetes mellitus on the fat and cholesterol contents of liver and spleen in the dog.* Acta physiol. scand. 1960. *49.* 29—34. — The normal fat content of dog liver was found to be 10 % of the dry weight; that of spleen, 5.3 %. Liver fat increased to 15 % in obesity, 21 % in pyometra and 58 % in diabetes mellitus; spleen fat increased significantly only in obesity. The water content of liver decreased very slightly in obesity and considerably in diabetes mellitus; that of spleen was not affected by any of the conditions studied. The normal cholesterol content of dog liver was about 1,000 mg per 100 g of fat-free dry tissue whereas that of spleen was about 1,700. Neither liver nor spleen cholesterol was significantly altered in obesity or pyometra, but in diabetes mellitus the cholesterol contents of both organs decreased by more than 30 %. These findings were interpreted as further evidence of the interrelationship of diabetes mellitus, obesity and pyometra in the dog.

[1] Postdoctoral Research Fellow of the National Cancer Institute, U. S. Public Health Service. Present address: Division of Biologics Standards, National Institutes of Health, Bethesda, Md., U. S. A.

[2] Present address: Department of Pathology, Kansas State College, Manhattan, Kansas, U. S. A.

A previous statistical survey by KROOK, LARSSON and ROONEY (1959) indicated an interrelationship of obesity, pyometra and diabetes mellitus in the dog. The present investigation was undertaken to study the relationship between fat and water contents of the liver and spleen in dogs suffering from these diseases. In a number of cases determinations of liver and spleen cholesterol were also made.

Material and Methods

The material was obtained from routine canine necropsies at the Department of Pathology, Kungl. Veterinärhögskolan, Stockholm 51, in 1957 and 1958. A total of 123 dogs was investigated. The material was divided into five groups with respect to the nutritional state and the diseases in question:

I. *Average:* normal subcutaneous fat deposition

II. *Good:* subcutaneous fat deposition above normal, but not fulfilling the criteria of obesity

III. *Obesity:* very marked increase of subcutaneous fat, intramuscular fat deposition and grossly visible lipemia

IV. *Pyometra:* chronic purulent endometritis

V. *Diabetes mellitus:* This diagnosis was made where lesions pathognomic of diabetes mellitus were proved, clinically (hyperglycemia and glucosuria) and/or by necropsy (pancreatic lesions — degranulation of beta cells, atrophy of islets, etc.).

The group designated obese excluded any dogs with pyometra or diabetes, even though they may have fulfilled the criteria of obesity.

The water and fat contents were determined by drying weighed slices of liver and spleen in an oven at about 40°C for 48 hours (until constant weight was reached). The dried tissue was then extracted in a Soxhlet apparatus with petroleum ether for 24 hours, the petroleum ether evaporated, and the fat weighed. In some instances chloroform and acetone were also used; values obtained were the same as when petroleum ether was the extraction fluid.

For sterol analyses, livers and spleens were extracted with acetone for 24 hours in a Soxhlet apparatus. Aliquots were saponified and determinations of total (*i.e.*, esterified + unesterified) sterol were carried out by the modified Moore-Baumann method (MOORE and BAUMANN 1952, FINLAYSON 1957), samples being read in 1 cm cuvettes in a Unicam (Unicam Instruments, Cambridge, England) spectrophotometer. Since no "fast-acting" (*i. e.*, Δ^7-unsaturated) sterols were detected in any of the samples, cholesterol values could be calculated directly using the formula: mg cholesterol/aliquot = $1.53 \times L_{620m\mu}$, where $L_{620m\mu}$ represents the optical density of the sample 38 min after addition of the reagents. Results were expressed as milligrams of cholesterol per 100 g fat-free dry weight of tissue.

In the histological examinations for fat Scharlach-Red stain was used on frozen sections of 6 μ thickness.

For statistical analyses the t-test was employed. The following symbols have been used:

* almost statistically significant (at the 5 % level)
** statistically significant (at the 5 % level or better)
*** highly statistically significant.

Table I. Fat and water content of liver and spleen in different nutritional conditions, obesity, pyometra and diabetes mellitus

	g fat/100 g dry tissue[1]				g water/100 g fresh tissue[1]			
	n[2]	Liver	n	Spleen	n	Liver	n	Spleen
Average ..	64	10.0 ± 0.66	41	5.3 ± 0.33	64	72.7 ± 0.41	48	75.7 ± 0.36
Good	27	10.3 ± 0.96	23	5.6 ± 0.59	27	73.2 ± 0.64	24	76.0 ± 0.38
Obesity ..	20	15.5 ± 1.68**	13	6.8 ± 0.62**	20	71.6 ± 0.55	14	76.2 ± 0.69
Pyometra	6	21.0 ± 5.81*	5	10.2 ± 3.72	6	72.2 ± 1.00	5	76.0 ± 0.47
Diabetes mellitus..	6	58.4 ± 8.32***	3	7.3 ± 1.69	6	57.2 ± 3.46***	2	81.1 ± 6.45

[1] All values are means ± standard error of mean; asterisks refer to statistical significance of difference from "Average" group.

[2] Number of samples analyzed.

Results

Preliminary statistical analyses of the results of liver and spleen fat determinations showed no significant differences between the sexes, a somewhat surprising finding in view of the higher incidence of obesity in females (KROOK *et al.* 1959). Therefore the data obtained from both sexes were combined for presentation in Table I. The normal fat content of liver was 10 % of the dry weight, the value being the same for average and good nutritional states. In obesity the fat content of the liver increased to 15 %; in pyometra, to 21 %; in diabetes mellitus, to 58 %. The normal fat content of the spleen, about 5.3 %, was significantly increased only in obesity.

The normal water content of liver, about 73 %, was not affected by the nutritional state or by pyometra. In obesity there was a very slightly decreased water content, and in diabetes mellitus the decrease became highly statistically significant (Table I). On the other hand, the normal water content of spleen, about 76 %, was not significantly changed in any of these conditions.

The livers and spleens of 32 of the dogs from the above study were analyzed for cholesterol. Liver and spleen fat contents of the various groups within this sample were similar to those in Table I, indicating that these animals were representative of their respective conditions. Moreover, as in the case of total fat, preliminary statistical analyses showed that the slight elevations of cholesterol levels in livers and spleens of females were not significant, so data from both sexes were combined. The normal cholesterol content of dog liver was found to be about 1,000 mg per 100 g of fat-free dry tissue; that of spleen, about 1,700 mg per 100 g fat-free dry tissue (Table II). There was a fairly close correlation between the cholesterol contents of the two organs; the normal spleen/liver ratio was not significantly changed by any of the nutritional or disease states in-

Table II. Cholesterol content of liver and spleen in different nutritional conditions, obesity, pyometra, and diabetes mellitus

	No. samples	mg cholesterol/100 g fat-free dry tissue[1]		mg cholesterol/10 g fat[1]	
		Liver	Spleen	Liver	Spleen
Average	[2]8, 10	945 ± 122	1,650 ± 97	1,620 ± 334	3,410 ± 338
Good	9	1,080 ± 132	1,830 ± 201	1,250 ± 333	3,540 ± 341
Obesity	8	1,210 ± 107	1,950 ± 125	690 ± 129**	2,710 ± 326
Pyometra ..	2	1,230 ± 293	1,670 ± 800	823 ± 208	2,090 ± 185**
Diabetes mellitus	3	650 ± 60*	1,120 ± 172**	46 ± 10***	1,800 ± 824

[1] All values are means ± standard error of mean; asterisks refer to statistical significance of difference from "Average" group.

[2] Eight samples of liver, ten samples of spleen.

vestigated. Neither spleen nor liver cholesterol was significantly altered by a change in nutritional state, obesity or pyometra; however, in diabetes mellitus the cholesterol contents of both organs were decreased by more than 30 % (Table II).

In the spleen the cholesterol content followed the level of total fat quite closely, while in the liver no such relationship was apparent. When the results were expressed as mg cholesterol per 10 g of fat, the value for liver cholesterol was significantly decreased in obesity and very significantly decreased in diabetes mellitus (Table II). The decrease in liver cholesterol in pyometra was not statistically significant. Values for spleen cholesterol expressed on this basis were not greatly affected by nutritional state or disease, only that in the case of pyometra being significantly decreased.

Histological examination of the livers of 52 of the dogs substantiated the results of the total fat analyses. Liver fat deposition of the degenerative type was not demonstrable, only 6 of 22 livers from the "average" group showing even a low degree of fat. This situation was not appreciably altered in the nutritionally "good" dogs, but in obesity low or moderate fat deposition was seen in 9 of 12 livers. In diabetes mellitus the results were striking; all livers showed a high degree of fat deposition (Table III).

Discussion

In a statistical analysis of about 11,000 canine necropsies, including approximately 1,000 cases of uncomplicated obesity, 500 cases of pyometra, and 170 cases of diabetes mellitus, KROOK *et al.* (1959) found these diseases to be closely interrelated as far as the sex and the breed distribution, as well as many of the

Table III. Histological examination of liver for fat in different nutritional conditions, obesity, pyometra and diabetes mellitus

	No. samples	Degree of fat in liver cells			
		0	+	++	+++
Average	22	16	6	0	0
Good	10	6	4	0	0
Obesity	12	3	5	4	0
Pyometra	2	1	1	0	0
Diabetes mellitus ..	6	0	0	0	6

anatomical lesions, were concerned. The common denominator of these lesions (except those considered specific characteristics of pyometra and of diabetes mellitus) was the tendency toward fatty infiltration in various organs. Moreover, these authors found statistical evidence that obesity very likely precedes pyometra and diabetes mellitus.

Obesity in the dog is predominant in females and in certain breeds, and it appears unlikely that this sex and breed distribution can be explained on the basis of simple overfeeding alone. However, regardless of its origin, which is probably multiple, obesity once induced is likely to exert extra stress upon the endocrine system. As a result of this stress a polyendocrine syndrome including pyometra and diabetes mellitus could be induced. KROOK *et al.* (1959) have designated this the DOP-syndrome.

If, then, the DOP-syndrome represents a series of progressive changes involving fat deposition, it could be expected that a progressive deposition of fat would occur from the earlier to the later stages of the syndrome. That is, if obesity were the first disease in the sequence, one could expect an increased fat deposition in obesity and still more pronounced fatty infiltration in pyometra and diabetes mellitus. Data from our studies of liver fat (Tables I and III) have shown this to be the case. Liver fat contents were: normal 10 %, obesity 15 %, pyometra 21 %, and diabetes mellitus 58 %, with histological examinations revealing similar trends. The authors interpret these biochemical and histological findings as further evidence that there does exist an intimate relationship between obesity and pyometra and diabetes mellitus in the dog, and that obesity precedes the other two conditions.

Despite the varying dietary histories of the dogs, the cholesterol contents of liver and spleen, expressed as mg per 100 g of fat-free dry tissue, were quite similar to those reported for rats fed a stock ration (WELLS and BAUMANN 1954). When the results of liver fat and cholesterol determinations were combined and expressed as mg cholesterol per unit weight of fat, the values obtained were significantly lower in obesity and very much lower in diabetes mellitus

(Table II). This indicates that in obesity the excess lipid deposited in the liver is poorer in cholesterol than is the normal liver fat. In diabetes mellitus the excess must also be poor in cholesterol, but there is an absolute decrease in cholesterol as well. Whether this can be taken as evidence for a "washing out" of cholesterol by the tremendous influx of fat cannot be said without further study, but such a suggestion seems tenable in view of the observation of PIHL (1955) that high dietary fat promoted the disappearance of cholesterol from the liver of the rat.

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Acta physiol. scand. 1960. 49. 35—41

Aus der Klinisch-physiologischen Abteilung des Karolinska Sjukhuset, Stockholm, Schweden

Hämoglobinmenge und Blutvolumen der Ratte und ihre Beziehung zum Körpergewicht

Von

BERNHARD TRIBUKAIT

Eingegangen am 14 Oktober 1959

Abstract

TRIBUKAIT, B., *Hämoglobinmenge und Blutvolumen der Ratte und ihre Beziehung zum Körpergewicht.* Acta physiol. scand. 1960. *49.* 35—41. — The total amount of haemoglobin and blood volume in the normal male rat has been estimated by a modified CO-method and related to body-weight. With increasing body-weight the total amount of haemoglobin decreases from 0.8 g Hb/100 g body-weight in the rat of 100—150 g to 0.68 g Hb/100 g body-weight in the rat heavier than 450 g, and the blood volume decreases from 0.85 to 0.67 ml/100 g body-weight. The results are compared with those of other workers and are discussed.

Über das Blutvolumen der männlichen Ratte in Abhängigkeit von Alter und Körpergewicht ist von GARCIA (1957) in einer ausführlichen Arbeit berichtet worden. Die Resultate dieser mit Hilfe radioaktiv gezeichneter Erythrocyten (Fe^{59}) durchgeführten Untersuchungen stimmen mit den von anderen Autoren gefundenen Ergebnissen, die ebenfalls eine Zellverdünnungsmethode verwendet haben, im ganzen überein (BERLIN *et al.* 1949, SHARPE, CULBRETH und KLEIN 1950, MUEHLHEIMS, DELLENBACK und RAWSON 1959).

Absolutwerten kommt man wegen der Differenzen zwischen Körperhämatocrit und venösem Hämatocrit oder auch aus rein methodologischen Gründen mit dieser wie mit irgendeiner anderen Methode nur nahe. In den meisten Fällen will man jedoch auch nur unter bestimmten experimentellen Bedingungen erhaltene Resultate mit Normalwerten vergleichen und ist weniger an Absolutwerten interessiert. Abgesehen davon, dass unterschiedliche

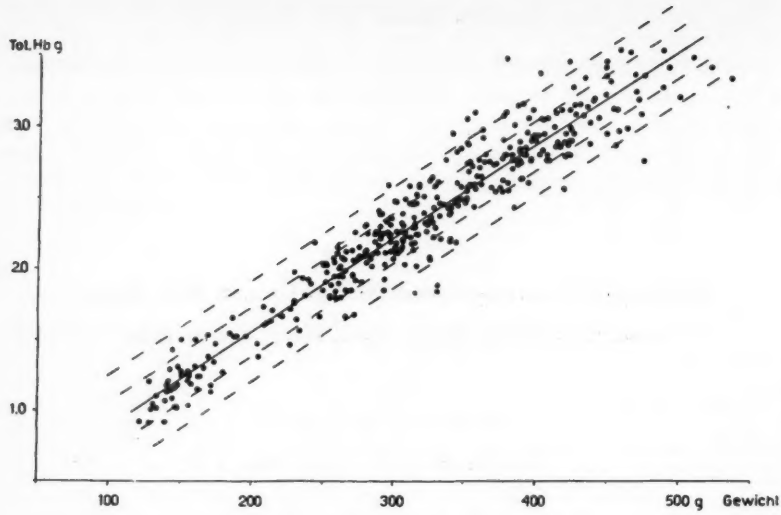

Abb. 1. Verhalten der Gesamthämoglobinmenge (Ordinate) zum Körpergewicht (Abszisse). Gleichung der Regressionslinie (ausgezogene Linie): $y = 0.0066\,x + 0.24$. $r = 0.96$. Gestrichelte Linien = einfache bezw. doppelte Standardabweichung.

Lebensverhältnisse und Tierstämme unterschiedliche Blutvolumina erwarten lassen, können deshalb mit verschiedenen Methoden gewonnene Werte nicht ohne weiteres miteinander verglichen werden.

In der nachfolgenden Arbeit sollen das Normalverhalten der Hämoglobinmenge und des Blutvolumens in Abhängigkeit vom Körpergewicht der Ratte neben Hämoglobinkonzentration und Hämatocrit behandelt werden. Dadurch soll später die Beurteilung von Daten erleichtert werden, die unter verschiedenen experimentellen Bedingungen mit einer Methode zur wiederholten Bestimmung der Hämoglobinmenge und des Blutvolumens gewonnen wurden.

Methodik

Die Untersuchungen wurden an männlichen Ratten eines Stammes (Wistar) während einer Zeit von etwa 2 Jahren ausgeführt. Die Tiere erhielten ad libitum Wasser, Mohrrüben und ein Rattenbrot folgender Zusammensetzung: Trockenmilch 3 %, Luzernenmehl 3 %, Fischmehl 3 %, trockene Brauhefe 3 %, Weizenkeime 6 %, Leinsaatkuchenmehl 6 %, Fleischmehl 5 %, Maismehl 20 %, Mischkornschrot 50 %, jodhaltiges Kochsalz 0.3 %, Futterkalk 1 %, Vitamin A 1.25 mill. IE/100 kg, Vitamin D_3 250,000 IE/100 kg.

Die Gesamthämoglobinmenge wurde mit einer Modifikation der sogenannten alveolaren CO-Methode bestimmt, und das Blutvolumen aus dem Gesamthämoglobin

Tab. I. Gesamthämoglobin/100 g Körpergewicht von Ratten verschiedener Gewichtsklassen

Gewicht g	Zahl	g Hb/100 g Körpergewicht
100—150	23	0.80±0.016
151—200	28	0.80±0.014
201—250	24	0.76±0.013
251—300	80	0.75±0.006
301—350	88	0.73±0.006
351—400	67	0.74±0.006
401—450	48	0.71±0.006
über 451	20	0.68±0.010

und der Hämoglobinkonzentration des Schwanzblutes × 0.75 berechnet. 0.75 ist ein Umrechnungsfaktor für den Körperhämatocrit. Bei dem Hämatocrit ist der in der Blütkörperchenfraction verbleibende Plasmaanteil berücksichtigt (Einzelheiten der Methoden siehe TRIBUKAIT 1959).

Ergebnisse

Das Verhalten der Hämoglobinmenge in Abhängigkeit vom Körpergewicht ist aus Abb. 1 ersichtlich. Von 378 Tieren ist die Hämoglobinmenge (Mittel von Doppelbestimmungen) auf der Ordinate gegen das Gewicht auf der Abszisse aufgetragen worden. Mit steigendem Gewicht wächst das Gesamthämoglobin, beide Grössen stehen in einer engen Beziehung zueinander (Korrelationskoeffizient = 0.96).

Wie aber weiter aus Tab. I hervorgeht, in der für die verschiedenen Gewichtsklassen das Gesamthämoglobin/100 g Körpergewicht berechnet ist, fällt mit steigendem Gewicht die Gesamthämoglobinmenge relativ ab. Das 1—2 Monate alte und 100—150 g schwere Tier hat 0.8 g Hb/100 g Körpergewicht, während die 1 Jahr alte Ratte mit über 450 g Gewicht nur noch 0.68 g Hb/100 g Körpergewicht hat.

Blutvolumenwerte stehen von 241 Tieren zur Verfügung. Entsprechend Abb. 1 ist in Abb. 2 das Blutvolumen auf der Ordinate gegen das Gewicht auf der Abszisse aufgetragen worden. Der, verglichen mit Gesamthämoglobin, für die Beziehung Blutvolumen zum Körpergewicht etwas geringere Korrelationskoeffizient von 0.87 dürfte zum grössten Teil auf den grösseren Methodenfehler zurückzuführen sein.

Das Blutvolumen/100 g Körpergewicht fällt ebenfalls wie das Gesamthämoglobin/100 g Körpergewicht mit steigendem Totalgewicht ab (s. Tab. II). Gegenüber 8.5 ml Blut/100 g Körpergewicht bei unter 200 g schweren Tieren haben über 400 g schwere Ratten nur noch 6.7 ml/100 g Körpergewicht.

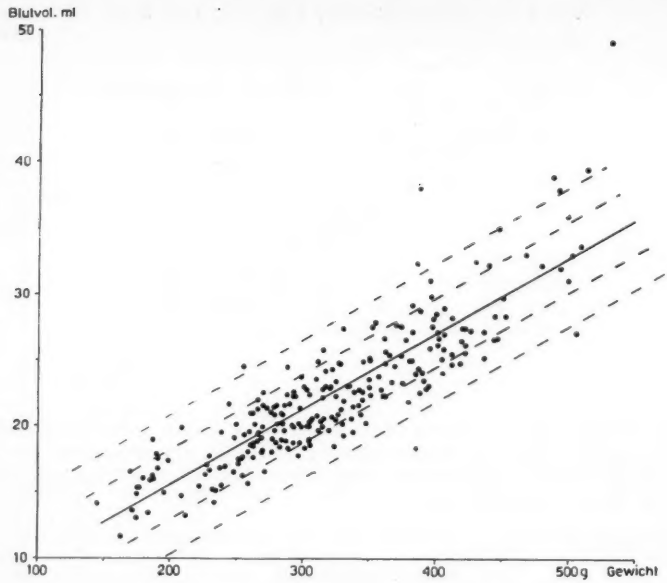

Abb. 2. Verhalten des Blutvolumens (Ordinate) zum Körpergewicht (Abszisse). Gleichung der Regressionslinie (ausgezogene Linie): $y = 0.057\,x + 4.02$. $r = 0.87$. Gestrichelte Linien = einfache bezw. doppelte Standardabweichung.

Hämoglobinkonzentration, Hämatocrit und die Erythrocyten-Hämoglobinkonzentration (g Hb/100 ml Erythrocyten) sind aus Tab. III ersichtlich. In Übereinstimmung mit GARCIA's Werten (1957) sind Hämoglobinkonzentration und Hämatocrit von Tieren unter 200 g gegenüber 100 g schwereren niedriger ($p < 0.001$); die Erythrocyten-Hämoglobinkonzentration unterscheidet sich jedoch nicht signifikant ($p = 0.4$—0.3).

Tab. II. Blutvolumen/100 g Körpergewicht von Ratten verschiedener Gewichtsklassen

Gewicht g	Zahl	ml Blutvol./100 g Körpergew.
unter 200	19	8.5±0.20
201—300	83	7.3±0.08
301—400	99	6.8±0.07
über 400	40	6.7±0.11

Tab. III. Hämoglobinkonzentration, Hämatocrit und Erythrocyten-Hämoglobinkonzentration (g Hb/100 ml Erythrocyten) von Ratten verschiedener Gewichtsklassen

Gewicht g	Zahl	Hb g%	Hct. %	$\frac{\text{Hb g\%}}{\text{Hct. \%}} \times 100$
100—200	22	13.6±0.37	45.6±0.96	29.8±0.50
201—300	55	14.8±0.16	48.9±0.37	30.3±0.28
301—400	80	15.1±0.11	49.3±0.38	30.7±0.19
über 401	29	14.6±0.17	47.5±0.45	30.8±0.27

Diskussion

Bestimmungen des Gesamthämoglobins der Ratte mit CO liegen nur in geringem Umfang vor. SCOTT und BARCROFT (1924) fanden bei einem Gesamtkörpergewicht von 124 g 0.822 g Hämoglobin/100 g Körpergewicht, GEMZELL und SJÖSTRAND (1954 a, b, 1956, 1958) bei 150—300 g schweren Tieren zwischen 0.97 und 0.68 g Hämoglobin.

Die mit der eigenen Methode gefundenen Werte liegen für die entsprechenden Gewichtsklassen zwischen 0.8 und 0.73 g/100 g Körpergewicht, d. h. teilweise niedriger und teilweise höher als bei den genannten Autoren. Den Stillstand der Hämoglobinmenge bei Erreichen von 250 g Körpergewicht, den SJÖSTRAND und GEMZELL (1954 a) in einer Versuchsserie fanden und der dem Verhalten des Menschen nach Abschluss des Längenwachstums entsprechen könnte (SJÖSTRAND 1949), wurde nicht beobachtet.

Neben dem Gesamterythrocytenvolumen ist mit radioaktiv gezeichneten Erythrocyten die Gesamthämoglobinmenge ohne die Schwierigkeiten, die die Differenzen zwischen Körperhämatocrit und Hämatocrit des Zentralblutes mit sich bringen, zu berechnen. Die von VAN DYKE *et al.* (1954) angegebenen Werte zwischen 0.75 g Hb/100 g Körpergewicht für das 170 g schwere Tier und 0.60 g Hb/100 g Gewicht für das 450 g schwere Tier liegen etwa 10 %, die von GARCIA (1957) angegebenen Werte von 0.64 bis 0.57 g/100 g Körpergewicht etwa 20 % unter den eigenen Werten. Diese Unterschiede sind erheblich grösser, als dass sie sich durch Aufnahme von CO durch Myoglobin oder durch im Knochenmark fixiertes Hämoglobin erklären liessen (TRIBUKAIT 1959) und mögen teilweise auf Verschiedenheiten der Tiere zurückzuführen sein.

Ist die Genauigkeit der Bestimmung der Hämoglobinmenge nur abhängig von der verwendeten Methodik, muss für das Blutvolumen der Körperhämatocrit berücksichtigt werden. Das gilt gleichermassen für eine Methode, bei der man mit radioaktiv gezeichneten Erythrocyten oder CO arbeitet, oder mit der man das Plasmavolumen misst. In den vorliegenden Untersuchungen wurde die Hämoglobinkonzentration vom Schwanzblut, die um ca. 12 % über dem des Zentralblutes liegt, bestimmt und dieser Wert mit 0.75 multi-

pliziert. Das Verhältnis Körperhämatocrit/Hämatocrit des Zentralblutes beträgt dann etwa 0.85 — ein Wert, der mit dem von EVERETT, SIMMONS und LASHER (1956) für die Ratte angegebenen übereinstimmt.

HUANG und BONDURANT (1956) fanden ein Verhältnis von 0.986, WANG (1959) ebenso wie FRIEDMAN (1959, bei der Maus) von 0.73.

Hinsichtlich der Blutverteilung bedeutet ein Verhältnis Körperhämatocrit/Zentralbluthämatocrit von 0.986, dass entweder Erythrocyten- und Plasmaanteil des Blutes praktisch unabhängig vom Durchmesser der durchströmten Gefässe sind — Untersuchungen von FÅHRAEUS (1929) stehen jedoch im Widerspruch zu einer solchen Auffassung — oder dass gewisse Gewebsabschnitte wie die Milz einen sehr hohen Hämatocrit haben müssen. Nach Splenektomie fiel auch tatsächlich der Faktor auf 0.945 ab.

WANG (1959) fand nach Splenektomie unvermindert eine Verhältniszahl von 0.73. Bei einem von der Autorin angegebenen Blutvolumen von 16.3 ml, einem Körperhämatocrit von 37.7 % und einem Zentralbluthämatocrit von 50.3 % beträgt das Gesamtplasmavolumen 10.22 ml und das Gesamterythrocytenvolumen 6.08 ml. Nimmt man nun mit GIBSON *et al.* (1956) an, dass ca. 20 % des Gesamtblutes im Kapillarsystem (+ Arteriolen und Venolen) liegt, so beträgt das Erythrocytenvolumen des Zentralblutes 8.2 ml, also mehr als überhaupt vorhanden ist. SJÖSTRAND (1935) hat die Blutmenge in sämtlichen peripheren Gefässen auf ca. 50 % der Gesamtblutmenge geschätzt. Da Milz und Lunge bei einem Hämatocrit von ca. 50 % davon 29 % einnehmen, entfallen auf das totale Gefässvolumen der vorwiegend energieverbrauchenden Gewebe 5.79 ml Blut mit 0.8 ml Erythrocyten. Auch dieser Wert erscheint unwahrscheinlich.

Diese Überlegungen zeigen, wie schwierig es zumindest bei kleinen Tieren ist, Absolutblutvolumina wirklich exakt zu bestimmen, selbst wenn gleichzeitig Plasma- und Erythrocytenvolumen wie bei diesen Untersuchungen gemessen werden, und wie zweifelhaft die Resultate solcher Untersuchungen sind. Gleichermassen können alle aus Erythrocytenvolumen, Hämoglobinmenge oder Plasmavolumen berechneten Blutvolumina nur als Näherungswerte aufgefasst werden.

Zusammenfassung

Hämoglobinmenge und Blutvolumen der normalen männlichen Ratte wurden mit einer modifizierten CO-Methode bestimmt und in Relation zum Körpergewicht gestellt. Mit steigendem Körpergewicht sinkt die Hämoglobinmenge von 0.8 g Hb/100 g Körpergewicht bei dem 100—150 g schweren Tier auf 0.68 g Hb/100 g Körpergewicht bei dem über 450 g schweren Tier, das Blutvolumen von 0.85 auf 0.67 ml/100 g Körpergewicht.

Die Resultate werden mit denen anderer Autoren verglichen und besprochen.

Mit Unterstützung durch Prof. T. Sjöstrand zur Verfügung gestellten Mitteln des »Therese och Johan Anderssons Minnesfond« und »Riksidrottsförbundets poliklinikkommitté«.

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From the Department of Pharmacology, Karolinska institutet, Stockholm, Sweden

Inhibitory Effects of Acid in Antrum-Duodenum on Fasting Gastric Secretion in Pavlov and Heidenhain Pouch Dogs

By

SVEN ANDERSSON

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Abstract

ANDERSSON, S. *Inhibitory effects of acid in antrum-duodenum on fasting gastric secretion in Pavlov and Heidenhain pouch dogs.* Acta physiol. scand. 1960. *49*. 42—56. — Exclusion of the antrum and duodenum was performed on Pavlov and Heidenhain pouch dogs. The gastrointestinal passage was re-established by gastrojejunostomy and a cannula inserted into the excluded parts. A fasting secretion[1] appeared. If hydrochloric acid or gastric juice was introduced into the antrum and duodenum the secretion was markedly inhibited. The inhibitory effects were equally pronounced in Heidenhain and in Pavlov pouch dogs. The results suggest that the inhibition is humorally mediated. As the stimulatory mechanisms behind the fasting secretion are unknown it is not possible to draw any definite conclusions concerning the exact nature of the inhibition.

The mechanism whereby the secretion of gastric juice is made to cease at the end of the digestive period is not known. It is assumed that some importance attaches to hydrochloric acid as a secretion-inhibitor. SOKOLOV (1904) showed that instillation of hydrochloric acid or gastric juice into the stomach or the duodenum of Pavlov pouch dogs inhibited the HCl secretion from the pouch. Many later writers (DAY and WEBSTER 1935, WILHELMJ, MCCARTHY and HILL 1937, OBERHELMAN *et al.* 1952, CODE and WATKINSON 1955) have since claimed that under certain conditions hydrochloric acid inhibits the production of

[1] Fasting secretion: Defined in this paper as the gastric secretion occurring in dogs despite 18—24 hours' starvation.

gastric juice on contacting the mucosa of the antrum and the duodenum. Others (STEVENS, SEGAL and SCOTT 1939) have disputed this.

It is not known whether the inhibition of gastric secretion produced by acid is of humoral or nervous character. Nor do we know whether the inhibitory effect is due to the activation of an inhibitory or to the blocking of a stimulatory mechanism.

Following exclusion of the antrum and duodenum in Pavlov pouch dogs, UVNÄS *et al.* (1956) and ANDERSSON, ELWIN and UVNÄS (1958) found augmented secretory responses to diverse stimuli. In more than half the animals there was also a fasting secretion.

In the present investigation such fasting secretion was induced experimentally in Pavlov and Heidenhain pouch dogs and the inhibitory effect of instillation of various acid solutions into the excluded parts studied.

Methods

The experiments were carried out on four adult mongrel dogs. In two of these (no. 6 and 75) Pavlov pouches were constructed by a method reported earlier (UVNÄS *et al.* 1956); in the other two (no. 18 and 19), Heidenhain pouches were created. Three to four weeks after the preparation of the pouches the dogs were subjected to a second operation. The antrum and duodenum were isolated from the rest of the stomach by a double mucosal wall between the corpus and the antrum, and the gastrointestinal passage was restored by anastomosis between the stomach and the first loop of jejunum. In the two Heidenhain pouch dogs also a cannula was inserted into the antrum (Fig. 1: III). In a third operation on one of the Pavlov pouch dogs (no. 6) the antrum was entirely separated from the remainder of the stomach by an incision running between the two previously constructed mucosal walls. At the same time a cannula was placed in the antrum. The mode of preparation is illustrated in Fig. 1: I. In the other Pavlov pouch dog (no. 75) the pylorus was divided and the duodenum closed. The antrum was brought out through the abdominal wall as a cutaneous fistula and a cannula was introduced into the duodenum about 10 cm distal to the pylorus (Fig. 1: II).

All operations were performed under pentobarbital sodium anesthesia (25—40 mg/kg i.v.). For the postoperative management and subsequent care of the dogs see UVNÄS *et al.* (1956).

The gastric secretory output was collected in 15-minute portions, the volume being recorded and the amount of total acid determined by titration of each portion against 0.1 N NaOH with phenolphthalein as indicator.

In each experiment the secretion was recorded for a control period of one to two hours prior to the instillation of various solutions into the antrum-duodenum.

The solutions used for instillation into the antrum-duodenum were hydrochloric acid, gastric juice (pH 1.1) and 0.9 per cent sodium chloride solution. In the case of dogs no. 6, 18 and 19 the solutions were instilled via a rubber catheter introduced into the antrum-duodenum through the antral cannula. In this way the instillate came into contact with both the antral and the duodenal mucosa before its passage out into the small intestine. In dog no. 75, with antrum and duodenum separated, the antrum was perfused via a two-way rubber catheter introduced through the antral fistula, an arrangement by which it was possible to drain the antrum and avoid increase of the perfusion pressure. For acid perfusion of the antrum 0.1 N HCl was used. Repeated

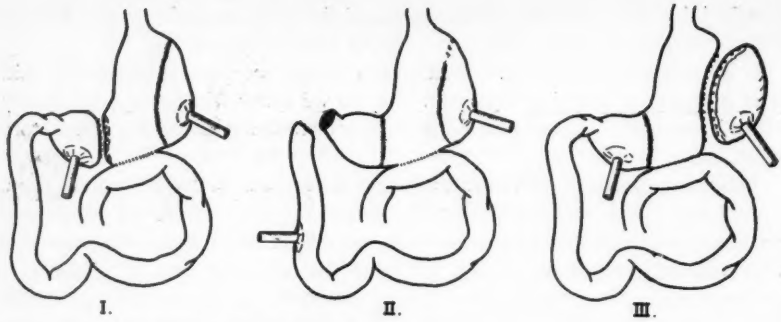

Fig. 1. Operative preparations of the dogs used:
I. (no. 6) Pavlov pouch; antrum totally isolated from the rest of the stomach; gastrojejunostomy and cannula in the antrum.
II. (no. 75) Pavlov pouch; mucosal wall between the corpus and antrum; pylorus divided and cutaneous fistula to the antrum; gastrojejunostomy and cannula in the duodenum.
III. (no. 18 and 19) Heidenhain pouch; mucosal wall between the corpus and antrum; gastrojejunostomy and cannula in the antrum.

pH determinations carried out on the perfusate showed that there was only a very slight rise in the pH of the perfused solution with the perfusion rates employed, *i. e.* about 100 ml per hour. The pH of the perfusate never exceeded 1.5.

Instillation into the duodenum of this dog was compassed by means of a rubber catheter inserted through the duodenal cannula and up to the proximal end of the duodenum. The instilled fluid was allowed to pass down into the small intestine. In those experiments on dog no. 75 in which the duodenal content was subjected to pH determinations a two-way catheter was used. About 20 ml was tapped via the draining opening of the catheter every 15 minutes and the pH determined electrometrically with a glass electrode. The samples were taken about 2 cm distal to the inflow of the instillate.

Prior to each experiment the dogs were fasted 18—24 hours.

Results

Inhibition of fasting secretion in Pavlov pouch dogs

A. *Instillation of acid into the antrum-duodenum*

In all the seven experiments performed on dog no. 6 (prepared according to Fig. 1: I) the instillation of hydrochloric acid or gastric juice into the antrum-duodenum caused inhibition of the fasting secretion.

Fig. 2 illustrates two experiments in which hydrochloric acid of varying acidity and volume was instilled. In experiment A, 0.1 N HCl was given at a rate of 45 ml per hour. The secretion was substantially reduced for two hours. Despite an unchanged instillation rate the secretion rose, during the last half-hour of instillation (period II). When the amount of acid was increased from 45 to 75 ml 0.1 N HCl per hour (period III) the secretion was once more depressed, though only temporarily. In experiment B, illustrated in the same

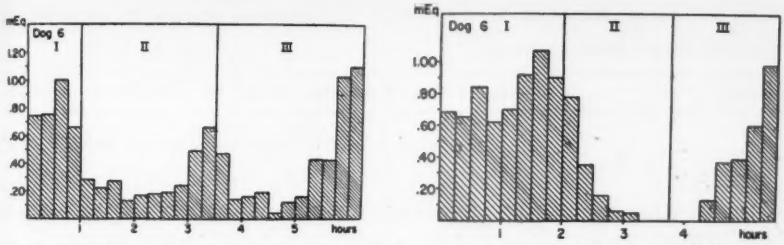

Fig. 2. Inhibition of fasting secretion in a Pavlov pouch dog by instillation of HCl into the antrum-duodenum.
A. I. Control. II. Instillation of 45 ml N/10 HCl per hour. III. Instillation of 75 ml N/10 HCl per hour.
B. I. Control. II. Instillation of 140 ml 15/100 N HCl per hour. III. Control.

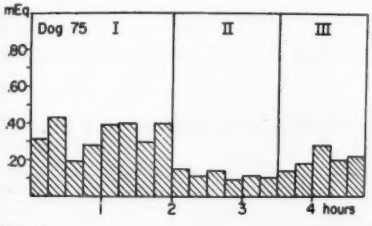

Fig. 3

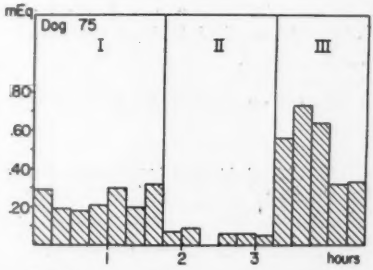

Fig. 4

Fig. 3. Inhibition of fasting secretion in a Pavlov pouch dog by perfusion of antrum with HCl.
I. Control. II. Perfusion of antrum with 100 ml N/10 HCl per hour. III. Control.
Fig. 4. Inhibition of fasting secretion in a Pavlov pouch dog by instillation of HCl into the duodenum.
I. Control. II. Instillation of 220 ml N/10 HCl per hour. III. Control.

figure, the secretion was totally inhibited by the instillation of 140 ml 0.15 N HCl per hour.

On instillation of gastric juice (pH 1.1) the inhibitory effect was just as good as that with pure hydrochloric acid (See Table I which also shows the other experiments performed on this dog).

In control experiments the instillation of 0.9 per cent sodium chloride solution into the antrum-duodenum was never observed to exert any inhibitory effects.

B. *Instillation of acid into the antrum or the duodenum*

In the hope of ascertaining whether the inhibitory effect of acid on the fasting secretion was elicited from the antrum or the duodenum or from both of them, a series of experiments were performed on dog no. 75. In this animal the antrum and the duodenum were surgically separated from each other (Fig. 1: II).

Table I. Effects of HCl, gastric juice and NaCl instillations into antrum-duodenum on fasting
(Experiments not shown in figures)

Experiment no.	Gastric secretion (mEq total acid): 15-minute collec-										
	1	2	3	4	5	6	7	8	9	19	11
	I				IIa						
1. IIa: 140 ml gastric juice (pH 1.1) per hour	0.60	0.62	0.74	0.70	**0.38**	**0.17**	**0.12**	**0.08**	**0.07**	**0.14**	**0.03**
IIb: 300 ml gastric juice (pH 1.1) per hour	I				IIa						IIb
2. IIa. 208 ml 0.9 per cent NaCl per hour	0.60	0.40	0.38	0.28	**0.40**	**0.63**	**0.75**	**0.93**	**1.08**	**0.92**	**0.59**
IIb: 220 ml N/10 HCl per hour											
	I				II						III
3. II: 152 ml N/10 HCl per hour	0.47	0.89	1.12	0.81	**0.44**	**0.25**	**0.05**	**0**	**0**	**0**	0
	I						II				
4. II. 144 ml 5/100 N HCl per hour	0.86	0.43	0.38	0.67	0.52	0.63	**0.29**	**0.16**	**0.10**	**0.22**	**0.20**
	I						II				
5. II: 136 ml 5/100 N HCl per hour	0.60	0.83	0.50	0.69	0.78	0.74	**0.34**	**0.16**	**0.26**	**0.12**	**0.21**

I. Control periods (pro-instillation).
II. Periods of instillation into antrum-duodenum.
III. Control periods (post-instillation).

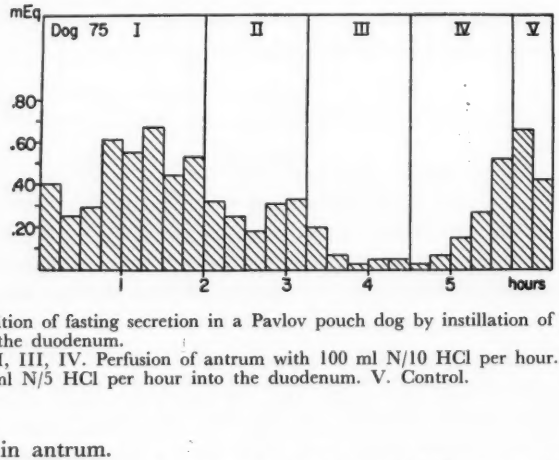

Fig. 5. Inhibition of fasting secretion in a Pavlov pouch dog by instillation of HCl into the antrum and the duodenum.
I. Control. II, III, IV. Perfusion of antrum with 100 ml N/10 HCl per hour. III. Instillation of 100 ml N/5 HCl per hour into the duodenum. V. Control.

a) Acid in antrum.

The effect of perfusion of antrum alone with 0.1 N HCl was studied in seven experiments. Fig. 3 shows one of these experiments. As may be seen, the secre-

gastric secretion in a Pavlov pouch dog (No. 6)

tion periods												Remarks
12	13	14	15	16	17	18	19	20	21	22	
IIa			IIb					III			
0.05	**0.93**	**0.01**	**0.01**	**0**	**0**	**0**	**0**	0	0.16	1.07	IIa: Almost total inhibition
											IIb: Total inhibition
IIb		III									
0.05	**0.02**	0.04	0.01	0.09	0.14	0.09	0.18	0.48			IIa: No inhibition
											IIb: Almost total inhibition
III											
0	0	0.09	0.20	0.42	0.58	0.33					II: Total inhibition
II				III							
0.47	**0.36**	**0.48**	**0.87**	0.85	0.83	1.24	0.80				II: Partial inhibition
II					III						
0.79	**0.42**	**0.72**	**0.62**	**0.57**	1.62	1.47	0.83	0.80			II: ~~Partial inhibition~~ III: Pronounced rebound effect

tion diminished somewhat during the perfusion of acid. In the majority of the experiments, however, doubtful or no inhibition was observed (Table II). A fact worth noting is that after antrum perfusion the gastric secretion frequently did not return — if it had fallen — to its initial level. Perfusion of antrum with sodium chloride solution had no inhibitory effect on the secretion (Table II).

b) Acid in duodenum.

The inhibitory effect of acid instillation into the duodenum alone was studied in six experiments. Fig. 4 presents data from one experiment with instillation of 220 ml 0.1 N HCl per hour. The secretion is here almost completely inhibited. In this experiment, as in several others, the inhibition was succeeded by a pronounced rebound effect, the post-inhibitory secretion rising above the pre-inhibitory level.

In the experiment shown in Fig. 5 the effects of HCl-instillation into the antrum and the duodenum have been compared. Perfusion of the antrum (period II) caused only an uncertain inhibition. There was no effective inhibition until acid was instilled also into the duodenum (period III). On the cessation of the duodenal instillation (period IV) the secretion rose to the control level (period I), despite the antral perfusion still going on.

Table II. Effects of HCl, gastric juice and NaCl installations into antrum or duodenum or both
(Experiments not shown in figures)

Experiment no	Gastric secretion (mEq total acid); 15-minute								
	1	2	3	4	5	6	7	8	9
A. *Acid in antrum*	I						II		
1. II: About 100 ml N/10 HCl per hour	0.25	0.36	0.44	0.20	0.30	0.31	**0.30**	**0.30**	**0.21**
	I						II		
2. II: About 100 ml N/10 HCl per hour	0.24	0.30	0.24	0.32	0.25	0.27	**0.14**	**0.31**	**0.24**
	I								
3. II: About 100 ml N/10 HCl per hour	0.45	0.52	0.64	0.68	0.71	0.90	**0.71**	**0.60**	**0.68**
	I						II		
4. II: About 100 ml N/10 HCl per hour	0.45	0.52	0.76	1.01	0.57	0.49	**0.34**	**0.20**	**0.21**
B. *Acid in duodenum*	I						II		
1. II: 120 ml N/10 HCl per hour	0.58	0.57	0.52	0.63	0.35	0.38	**0.26**	**0.16**	**0.10**
	I								
2. II: 100 ml gastric juice (pH 1.1) per hour (duodenal pH)	0.37	0.44	0.61 (7.6	0.16	0.19	0.29 7.7	0.40 7.7	**0.42 6.0**	**0.05 3.9**
	I								
3. II: 160 ml N/10 HCl per hour (duodenal pH)	0.31	0.42	0.40	0.28	0.34	0.26 (7.4	**0.29 2.4**	**0.20 2.5**	**0.12 2.3**
C. *Acid in antrum and duodenum*	I				IIa				
1. IIa+b: 100 ml N/10 HCl per hour in antrum IIb: 200 ml N/10 HCl per hour in duodenum	0.21	0.39	0.30	0.45	**0.19**	**0.09**	**0.13**	**0.11**	**0.18**
2. IIa + b: 65 ml N/5 HCl per hour in duodenum (duodenal pH) IIb: 100 ml N/10 HCl per hour in antrum	0.28 (7.7	0.20 7.7	0.24 7.6	0.27	0.27 7.6	**0.18 3.5**	**0.09 2.0**	**0.15 3.2**	**0.39 2.1**
D. *Controls with 0,9 per cent NaCl*	I								
1. II: 100 ml NaCl per hour in antrum	0.22	0.41	0.41	0.22	0.30	0.50	**0.36**	**0.62**	**0.72**
	I								
2. II. 150 ml NaCl per hour in duodenum	0.61	0.74	0.38	0.32	0.59	0.79	**0.48**	**0.16**	**0.31**

I. Control periods (pre-instillation).
II. Periods of instillation into antrum or duodenum.
III. Control periods (post-instillation).

Instillation of sodium chloride solution into the duodenum had no inhibitory effect on the secretion (Table II).

Comments: When instilled in sufficient amounts into antrum-duodenum of Pavlov pouch dogs hydrochloric acid or gastric juice inhibit the post-operative

on fasting gastric secretion in a Pavlov pouch dog (No. 75)

collection periods												Remarks
10	11	12	13	14	15	16	17	18	19	20	21	
II						III						
0.08	**0.24**	**0.14**	**0.18**	**0.23**	**0.18**	0.15	0.37	0.32	0.15	0.34		II: No inhibition
II			III									
0.13	**0.13**	**0.12**	0.10	0.09	0.12	0.13	0.13	0.18	0.18			II: No inhibition
II				III								
0.37	**0.29**	**0.28**	**0.39**	0.29	0.32	0.27	0.21					II: No inhibition
II			III									
0.27	**0.25**	**0.21**	0.36	0.39	0.39	0.31	0.40					II: Doubtful inhibition
		III										
0.11	**0.12**	0.24	0.38	0.29	0.53	0.64						II: Partial inhibition
II								III				
0.04	**0.02**	**0.01**	**0.09**	**0.19**	**0.31**	**0.07**	**0.14**	0.21	0.53	0.42	0.55	II: Partial inhibition
2.5	**2.2**	**5.0**	**2.2**	**2.3**	**2.2**	**2.2**	**6.5**	7.6	7.5)			
II							III					
0.11	**0.10**	**0.12**	**0.16**	**0.16**	**0.13**	**0.12**	0.32	0.51	0.44	0.44		II: Partial inhibition
2.7	**2.3**	**2.2**	**4.4**	**2.9**	**2.1**	**5.2**	6.1)					
			IIb				III					
0.20	**0.14**	**0.06**	**0.06**	**0**	**0**	**0**	0.04	0.04	0.17	0.30		IIa: Partial inhibition
												IIb: Partial inhibition
0.29	**0.13**	**0.21**	**0.06**	**0.16**	**0.10**	**0.04**	**0.10**	**0.10**	**0.09**	0.18	0.36	IIa: No inhibition
2.9	**3.2**	**2.0**	**2.0**	**1.7**	**1.8**	**1.8**	**2.1**	**2.5**	**2.2**	6.6	7.2)	
												IIb: Partial inhibition
II					III							
0.41	**0.55**	**0.35**	**0.46**	**0.39**	0.39	0.30	0.35	0.19	0.34	0.46		II: No inhibition
II					III							
0.30	**0.42**	**0.33**	**0.35**	**0.64**	0.52	0.34	0.45	0.53	0.63	0.39		II: No inhibition

fasting secretion. When the inhibitory effect of acid in surgically separated antrum and duodenum is compared, it becomes evident that the inhibitory effect of antral perfusion is rather weak and irregular, but that instillation into the duodenum causes effective inhibition of the secretion.

Table III. Effects of HCl and NaCl instillations into antrum-duodenum on fasting gastric secre-
(Experiments not shown in figures)

Experiment no.	Gastric secretion (mEq total acid); 15-minute								
	1	2	3	4	5	6	7	8	9
Dog no. 18.	I				IIa				
1. IIa: 75 ml N/10 HCl per hour IIb: 150 ml N/10 HCl per hour	0.87	1.19	1.33	1.24	**0.53**	**0.37**	**0.51**	**0.62**	**0.53**
	I								
2. II: 72 ml N/10 HCl per hour	0.18	0.18	0.45	0.26	0.37	0.17	0.23	0.23	**0.20**
Dog no. 19.	I								
1. II: 45 ml N/10 HCl per hour	0.93	0.77	0.80	0.67	0.90	0.79	0.91	0.61	**0.47**
	I							II	
2. II: 20 ml N/10 HCl per hour	0.81	1.29	1.24	0.98	1.20	1.30	1.16	**0.54**	**0.89**
Control with 0.9 per cent NaCl	I								
3. II: 100 ml NaCl per hour	0.09	0.14	0.09	0.10	0.13	0.18	**0.15**	**0.23**	**0.17**

I: Control periods (pre-installation).
II. Periods of instillation into antrum-duodenum.
III. Control periods (post-instillation).

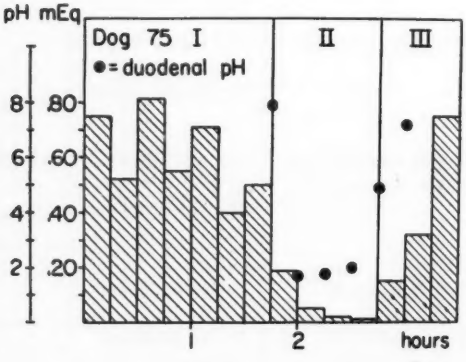

Fig. 6. Inhibition of fasting secretion in a Pavlov pouch dog by instillation of HCl into the duodenum. I. Control. II. Instillation of 100 ml N/5 HCl per hour. III. Control.

Duodenal pH during the instillation of acid

Owing to technical difficulties no satisfactory determinations of the intraduodenal pH were possible in dogs with an antral cannula alone (no. 6, 18 and 19); in dog no. 75, however, that had a duodenal cannula, this could be attained.

In five experiments on this latter dog regular determinations of the intraduodenal pH were made during acid instillation into the duodenum. These

tion in Heidenhain pouch dogs (No. 18 and 19)

collection periods												Remarks
10	11	12	13	14	15	16	17	18	19	20	21	
IIb							III					
0.35	**0.30**	**0.21**	**0.08**	**0**	**0**	**0**	0.04	0.12	0.21	0.26	0.70	IIa: Partial inhibition IIb: Total inhibition
II							III					
0.15	**0.14**	**0.04**	**0**	0	0	0	0.10	0.08	0.34	0.18		II: Total inhibition
II							III					
0.45	**0.17**	**0.08**	**0.09**	**0**	**0**	**0**	0.07	0.16	0.50	0.72	0.87	II: Total inhibition
II							III					
0.55	**0.66**	**0.62**	**0.30**	**0.48**	**0.66**	**0.57**	0.40	0.63	0.85	1.04		II: Partial inhibition
II					III							
0.14	**0.17**	**0.21**	**0.23**	**0.36**	0.18	0.29						II: No inhibition

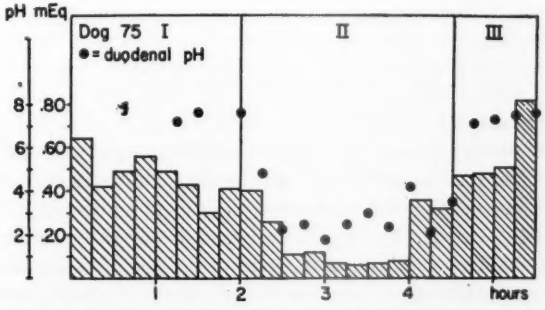

Fig. 7. Inhibition of fasting secretion in a Pavlov pouch dog by instillation of HCl into the duodenum.
I. Control. II. Instillation of 80 ml N/5 HCl per hour. III. Control.

experiments showed that in order to achieve a total blocking of the fasting secretion, the duodenal pH had to be reduced to about 2. This is illustrated in Fig. 6.

It was found that the pH tended to rise with prolonged instillation. This is demonstrated in Fig. 7. In this experiment, 0.2 N HCl was instilled, and the duodenal pH varied between 2 and 3 for the first two hours of the instillation

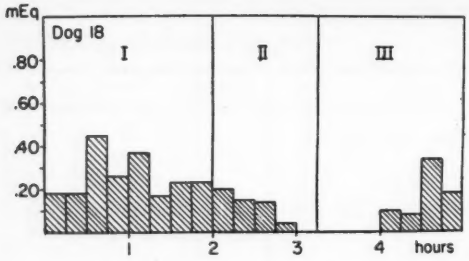

Fig. 8. Inhibition of fasting secretion in a Heidenhain pouch dog by instillation of HCl into the antrum-duodenum.
I. Control. II. Instillation of 120 ml N/10 HCl per hour. III. Control.

period, during which time the secretion was strongly inhibited. At the end of the period the pH rose quite abruptly to over 4, despite an unchanged instillation rate. There was a coincident increase in the secretion of acid. Similar behaviour was observed in other experiments (Table II).

Comments: Judging by the observations made in dog no. 75, there was to some extent a relation between the activity of the duodenal inhibitory mechanism and the intraduodenal pH. It therefore seems plausible to attribute the transient inhibitory effects observed in several experiments, *e. g.* those on dog no. 6 (Fig. 2A and experiments 4 and 5 in Table I), to a rise in the duodenal pH. The causes of such a rise in pH during acid instillation are obscure, but it might well be explained as due to emptying of the gallbladder and activation of the duodenal, hepatic and pancreatic secretions, all of which have a neutralizing action. It was noticed that the duodenal content became bile-stained when the pH rose.

Inhibition of fasting secretion in Heidenhain pouch dogs

Instillation of acid into the antrum-duodenum

The effects of HCl instillation into the antrum-duodenum were studied in five experiments. Fig. 8 gives data from one such experiment on dog no. 18 in which the fasting secretion was totally inhibited by the instillation of 120 ml 0.1 N HCl per hour. The secretion ceased 1—1$^1/_2$ hours after the start of instillation and returned 3/4 of an hour after the instillation was stopped.

In similar experiments on the two dogs relatively small amounts of acid were able effectively to inhibit the fasting secretion (Table III).

Instillation of sodium chloride had no effect on the secretion. Notwithstanding a relatively low fasting secretion (it seems evident from the other experiments that minor secretion is more easily inhibited than major secretion), and despite the instillation of 100 ml NaCl per hour, there was no inhibitory effect (Exp. 3 on dog no. 19, Table III).

Comments: The inhibitory action on the fasting secretion of HCl instillation into the excluded antrum-duodenum regions is equally pronounced in a Heidenhain and in a Pavlov pouch. In other words an intact vagal innervation is not a prerequisite for the inhibitory effects observed.

Discussion

The experiments reported above show that the instillation of hydrochloric acid or gastric juice into excluded antrum-duodenum regions has an inhibiting effect on fasting gastric secretion from as well Pavlov as Heidenhain pouches.

Inhibition induced from duodenum. Many writers have earlier observed that instillation of acid into the duodenum inhibits a current secretion of gastric juice. It is, however, still not clear whether the inhibitory mechanism activated by the acid is of humoral or a nervous character. Some writers, *e. g.* CODE and WATKINSON (1955) and SIRCUS (1958), assert that acid in the duodenum exercises its inhibitory effect via vagal fibers, as it has been possible to inhibit the secretion from Pavlov pouches, but not from Heidenhain pouches. On the other hand, observations have been made on Heidenhain pouch dogs which support the assumption of a humorally acting intraduodenal inhibitory mechanism activated by acid. Thus PINCUS *et al.* (1944) claim that it was possible to inhibit a postprandial secretion from a Heidenhain pouch by instillation of hydrochloric acid into the duodenum. GREENLEE *et al.* (1957) also assert that the duodenal inhibitory mechanism is of humoral character. These writers activated the secretion from a Heidenhain pouch by repeated instillation of "liver-solution" into an isolated antrum pouch. When a constant secretory level had been attained, the intraduodenal pH was reduced by giving the animal an acid test meal per os (pH 1). A pronounced inhibition of the secretion was the result.

The inhibitory effects of the instillation of acid into antrum-duodenum observed in the present investigation may very well be interpreted as due to the activation of an intraduodenal pH-sensitive mechanism. As the inhibition was equally pronounced in the Heidenhain pouch dogs, this favours the assumption of a humoral rather than a nervous mechanism. This with the proviso that inhibitory impulses to a Heidenhain pouch via sympathetic nerves can be ruled out. The significance of such nerves for the functioning of the acid glands is at present obscure.

Inhibition induced from antrum. In recent years many writers have claimed that also antrum is the seat of inhibitory mechanisms that may be activated by reduction of the antral pH. Information concerning the nature of the antral inhibitory mechanism is, however, at present very confused. Two theoretical possibilities have been suggested:

1. Gastrin is released from the antral mucous membrane on, inter alia, the chemical and mechanical stimulation of the latter. WOODWARD *et al.* (1954) and WOODWARD *et al.* (1957) have asserted that a low intra-antral pH inhibits this release of gastrin.

2. Several observations indicate that a secretion-inhibiting factor is released from the antrum at low antral pH. Thus STATE *et al.* (1955) were able to show that in dogs subjected to various gastric resections with and without retention

of the antrum and given daily injections of histamine in beeswax the incidence of ulcer was significantly lower in those dogs with the antra in situ. Furthermore, HARRISON, LAKEY and HYDE (1956), JORDAN and SAND (1957) and STATE and MORGENSTERN (1958), among others, have presented data pointing to the existence of a specific antral inhibitor released by acid.

In three of the animals in the present investigation acid has been instilled into both antrum and duodenum. In these experiments there was a total or pronounced inhibition of fasting secretion. The acid-perfusion of the antrum (dog no. 75, with the antrum and duodenum separated) produced only a slight and uncertain inhibition of the secretion, and it was only when acid was instilled also into the duodenum that the secretion could be effectively inhibited (Fig. 5). Also with acid instillation into the duodenum alone there was in most experiments a pronounced inhibition. It therefore seems justified to assume that the inhibitory effects observed in the three above-mentioned dogs when acid was instilled into both antrum and duodenum were mainly the result of activation of the duodenal inhibitory mechanism.

Stimulatory mechanisms behind the fasting secretion. It is, however, difficult to make direct comparisons between the secretory behaviour of the different animals, owing partly to different operative preparations and partly to the fact that the mechanisms behind the fasting secretion in the different animals are unknown. The prerequisites for the occurrence of a fasting secretion in these animals may be, inter alia:

1. Elimination of duodenal/antral inhibition due to alkalinization of the duodenal and antral regions, whereby earlier sub-threshold stimuli such as vagal impulses, gastrin etc. may be conceived to stimulate the parietal cells.

2. Release of gastrin. The antral cannulas might stimulate the antral mucosa. Mechanical stimulation of the antral mucosa causes liberation of gastrin (GROSSMAN, ROBERTSON and IVY, 1948). In our laboratory a pronounced increase of the fasting secretion has been observed to follow the insertion of antral cannulas in both Pavlov and Heidenhain pouch dogs (unpublished observations). If, moreover, the hypothesis advanced by WOODWARD *et al.* — viz., that a neutral to alkaline antrum milieu facilitates the release of gastrin — is correct, then there are in three of the animals favourable conditions for an increased output of gastrin from the antral mucous membrane.

One dog (no. 75) had no antral cannula, and the cause of the fasting secretion can in this case not have been mechanical stimulation of the antrum by a cannula. The acid-perfusion of the antrum produced, moreover, only a slight and uncertain reduction of the secretion. If the theory is correct that acid reaction in antrum blocks the gastrin release, this observation contradicts the view that the fasting secretion in dog no. 75 is due to a continuous release of gastrin from antrum. ANDERSSON *et al.* (1958) observed that antrum resection did not regularly eliminate the fasting secretion occurring after antrum-duodenum exclusion in Pavlov pouch dogs. On the other hand, WOODWARD

et al. (1954) were able to achieve a total blocking of the "spontaneous secretion" from an innervated total stomach pouch by perfusion of an isolated antrum pouch with 0.05 N HCl. It should nevertheless be borne in mind that in their experiments the antral pouch included some of the upper part of the duodenum. Their inhibitory effects might therefore be ascribed not to acid reaction in antrum but to acid reaction in the duodenum leading to activation of the duodenal inhibitory mechanism demonstrated in this paper.

Physiological significance of the duodenal inhibitory mechanism. In connection with the instillation of acid into the duodenum of dog no. 75 the intraduodenal pH was recorded. These experiments showed that an effective inhibition of the secretion did not occur until the duodenal pH had been reduced to about 2. The same has been observed by, amongst others, PINCUS *et al.* (1944), who did not get inhibition of secretion until the intraduodenal pH was below 2.5. These writers therefore questioned whether such low intraduodenal pH could be considered to lie within the physiological range. pH of the duodenal contents during the digestive period in dogs has been stated to fall, at the lowest, pH 3 (THOMAS and CRIDER 1936, BERK, THOMAS and REHFUSS 1942). These determinations have been carried out on duodenal samples taken relatively far down in duodenum, at least below the inflow of the bile and the pancreatic juice. It is possible that the corresponding pH-values for samples taken proximally from duodenum might be considerably lower. Moreover, at the end of the digestive period, when the food has left the stomach and the latter is secreting highly acid juice passing into the duodenum, it is possible that the pH of the duodenal contents reaches values of about pH 2.

Conclusions

The instillation of hydrochloric acid or gastric juice into excluded antrum-duodenum regions effectively inhibited an actual fasting secretion in as well Pavlov as Heidenhain pouch dogs. The acid-perfusion of an isolated antrum pouch produced only slight and uncertain inhibition of secretion, whereas on the other hand, the instillation of acid into the duodenum effectively inhibited the secretion. The inhibitory mechanism activated by acid instillation into the antrum-duodenum is therefore presumed to be localized mainly to the duodenum. Since the inhibitory effects were equally pronounced in Heidenhain and in Pavlov pouch dogs, this favours the assumption of a humoral rather than a nervous inhibitory mechanism acting on the HCl-glands.

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From the Department of Physiology, Karolinska Institutet, Stockholm, Sweden

The Effect of Catechol on the Isolated Guinea-pig Ileum

By

N. SJÖSTRAND

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Abstract

SJÖSTRAND, N. *The effect of catechol on the isolated guinea-pig ileum.* Acta physiol. scand. 1960. *49.* 57—61. — Catechol in doses of 0.5—5 mg, added to the isolated guinea-pig ileum in a 15 ml bath, was generally found to induce contraction of the strip. Atropine and paralysing doses of nicotine abolished this contraction. Catechol in doses of 5—10 mg added to the organ bath had a relaxing action on the ileum. The probable way of stimulation via the intestinal ganglia is discussed.

It has recently been shown that catechol (1,2-dihydroxybenzene) is a natural constituent of human urine (FIKER 1953, EULER and LISHAJKO 1959, SMITH 1959) where it occurs in considerable quantities in a readily hydrolysable conjugate. Some pharmacological properties of catechol have been studied by BARGER and DALE (1910—1911), who concluded that it appeared to be a general, but not powerful stimulant of plain muscle. A cardiostimulant effect of catechol has been demonstrated by WALTON, WALTON and THOMPSON (1959). Of considerable interest are the findings of BACQ (1936) who described a sensitizing action of catechol and other dihydroxybenzenes on the effect of adrenaline and the recent discovery that catechol inhibits the catechol O-methyl transferase (BACQ *et al.* 1959).

In view of the occurrence of catechol in normal urine and presumably in some tissues it appeared to be of interest to study some of its actions. The present report deals with some actions on the isolated guinea-pig ileum.

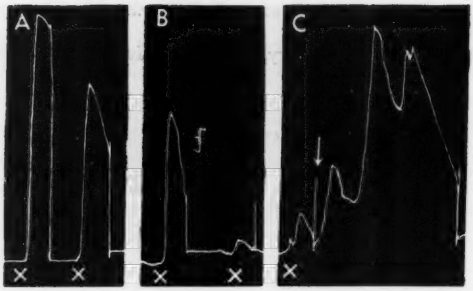

Fig. 1. Isolated guinea-pig ileum; bath volume 15 ml. All records from the same segment. At x addition of 2 mg catechol; at arrow washing. Tachyphylaxia, A and B, and "after effect", C.

Materials and methods

Young guinea-pigs weighing 150—250 g were killed by a blow on the head. A segment about 2 cm long of the distal part of the ileum was placed in a 15 ml organ-bath containing Tyrode solution aerated with oxygen and kept at 38° C. The contractions were recorded with a linear frontal writing lever.

The Tyrode solution had the following composition: 0.8 per cent NaCl, 0.02 per cent KCl, 0.02 per cent $CaCl_2$, 0.01 per cent $MgCl_2$, 0.1 per cent $NaHCO_3$, 0.005 per cent $NaH_2PO_4 \times H_2O$ and 0.1 per cent glucose.

The solutions used were: acetylcholine chloride 0.1 μg per ml, atropine sulphate 2.5 μg per ml, catechol 100 and 10 mg per ml in aqueous solution, histamine dihydrochloride 10 and 1 μg per ml, nicotine tartrate 1 and 0.1 mg per ml and substance P 60 units per ml.

Results

Catechol in doses smaller than 0.5 mg added to the 15 ml organ-bath had almost no effect; in doses of 0.5—5 mg it usually had a stimulating effect, the type and strength of which, however, varied considerably even in the same segment. The doses used sometimes produced a brief contraction of the intestine immediately followed by a relaxation or, occasionally, a rather slow increase in tone. In some experiments the tonic effect was accompanied by pendular movements or arose in a rather stepwise fashion. In almost every experiment the phenomenon of tachyphylaxia was seen, sometimes so pronounced that a new contraction due to catechol could not be produced for several minutes. For this reason there was no clear relation between dose and response (Fig. 1 a and b). In some cases a curious "after effect" was seen after washing the bath, consisting in pendular movements or a strong increase in tone. The latter often exceeded the response to the earlier catechol dose threefold or more. No such effect was observed after washing the bath after a contraction induced by acetylcholine, histamine or substance P (Fig. 1 c). Because of the

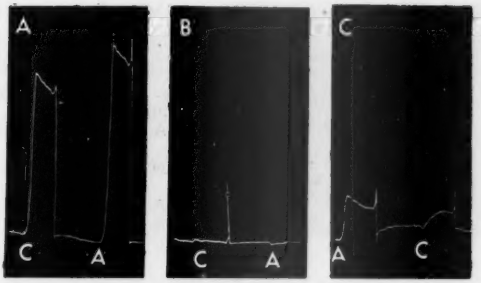

Fig. 2. Isolated guinea-pig ileum; bath volume 15 ml. A = 0.02 μg acetylcholine, C = 2 mg catechol.
A before addition of atropine
B 1 min after 5 min atropinization 1 : 2 mill.
C 8 min after atropinization.

irregular response to catechol, comparative data on its action are hard to present; in the most sensitive segment, however, the response to 2 mg catechol corresponded to 2 units of substance P and 0.005 μg acetylcholine, but it usually was of a lower magnitude.

Atropine 1 : 2 millions, sufficient to abolish the action of acetylcholine in the doses used while leaving the effect of substance P unimpaired, completely inhibited the stimulating effect of catechol as well as the "after effect". Moreover, this stimulating effect returned at the same time as that of acetylcholine (Fig. 2 a, b and c).

Paralysing doses of nicotine (210 μg), which totally obliterated the effect of a subsequent stimulating dose of nicotine (30 μg) but had no marked effect on the effect of acetylcholine, histamine and substance P, completely abolished the stimulation following catechol and the "after effect". (Fig. 3 a and b.)

Catechol in doses of 5—10 mg often had a slight stimulating effect but in some cases inhibited the intestine. In these doses catechol also inhibited contractions induced by acetylcholine, histamine and substance P (Fig. 4). This effect was not seen with the smaller stimulating doses, where an additive effect could be observed.

Discussion

It has been shown in these experiments that catechol possesses a double type of action on the guinea-pig intestine; thus in smaller doses the usual effect is stimulation but in larger doses catechol exerts an inhibitory action.

As far as the stimulating effect of catechol is concerned the question must arise whether this effect is a direct one due to catechol reacting with peripheral receptors or an indirect one mediated by some other factor released by catechol. Stimulation of smooth muscles induced by catechol has earlier been described

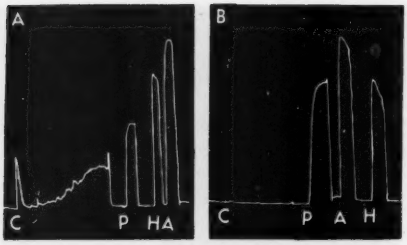

Fig. 3. Isolated guinea-pig ileum; bath volume 15 ml.
A = 0.01 μg acetylcholine, C = 0.5 mg catechol,
H = 0.1 μg histamine, P = 3 units substance P.
A before nicotine
B 210 μg nicotine in the bath.

Fig. 4. Isolated guinea-pig ileum; bath volume 15 ml.
A = 0.1 μg acetylcholine, H = 1 μg histamine,
P = 12 units substance P. At arrow addition of 10 mg catechol.

by BARGER and DALE (1910—1911), who suggested a direct tonic effect on the muscle fibres. In their report, however, no experiments were performed to prove that the stimulating property of catechol was due to a direct action on the muscle fibres. The fact that, in the present report, the stimulating property of catechol was abolished by atropine or nicotine indicates that catechol stimulates the intestine through liberation of acetylcholine and that this liberation is mediated by the ganglia in the walls of the intestine. Thus catechol in the doses used probably has an action like that of nicotine in small doses. The usually occurring tachyphylaxia could be explained if one assumes that catechol, like nicotine, has a secondary and persistent depressive action on the ganglia. Moreover an interference with the inhibitory action may complicate the picture. The "after effect" often seen could be explained by assuming a relatively strong affinity of catechol to the ganglia, causing small stimulating doses to persist in the ganglia after washing the bath. That the "after effect" has the same origin as the direct stimulating effect is probable, since it too never occurred after atropinization or nicotine paralysis.

The inhibitory action of catechol in larger doses seems to be of a general type, since it could be shown after contractions due to such different stimulating substances as acetylcholine, histamine and substance P. A depressant action of catechol on heart muscle has been described by GATGOUNIS and WALTON (1959), who further found the cardiostimulating action of catechol to be of central origin. Recently the general spasmolytic property of phenol and some of its derivates has been investigated by JAQUES and DOEPFNER (1959). Catechol was not tested but its isomers hydroquinone (1,4-dihydroxybenzene) and

resorcine (1,3-dihydroxybenzene) were found to possess inhibitory properties of a generalized kind. Furthermore, since ortho-, meta-, and para cresoles differed very little in spasmolytic effect, the relaxing property probably is shared by most phenols.

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From the Research Laboratories of the State Alcohol Monopoly, Helsinki, Finland

A Method for Recording General Activity of Mice

By

ALPO ARVOLA

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Abstract

ARVOLA, A. *A method for recording general activity of mice.* Acta physiol. scand. 1960. *49.* 62—64. — The method is based on the movements of a mouse on the labile floor of the cage. The labile floor rests on a vertical metal axle in the center. Telephone exchange relays at the bottom in the center of each wall register the movements of the floor. The method is suitable for measuring activity during short periods, because the apparatus registers all movements of the mouse.

There are two main types of recording devices for registration of the activity of small mammals: the revolving drum in which activity is measured as number of revolutions and consequently distance traversed (STEWART 1898), or cages with a labile floor resting on some kind of mechanical, pneumatic or electric device for registration of movements of the floor (SZYMANSKI 1914).

For a study on the effects of alcohol on the activity of mice, an apparatus was required which would register as accurately as possible all movements of the animals. The revolving drum gives a record of walking and running only. General activity also includes movements not involving locomotion such as preening and scratching. These will not be recorded by the revolving drum, or will result in a recorded distance which does not at all correspond to the energy expenditure involved. Thus an apparatus with registration of floor movements appeared more suitable for the present purpose. The final design is similar in principle to that described by RICHTER (1927). The present activity cage was constructed especially for use in brief experimental periods (1—2 hr.).

The square cage is made of plywood with a glass inserted in one wall for observation (Fig. 1). At the bottom in the center of each wall there is an opening (15—20 mm) for the registering telephone exchange relays which

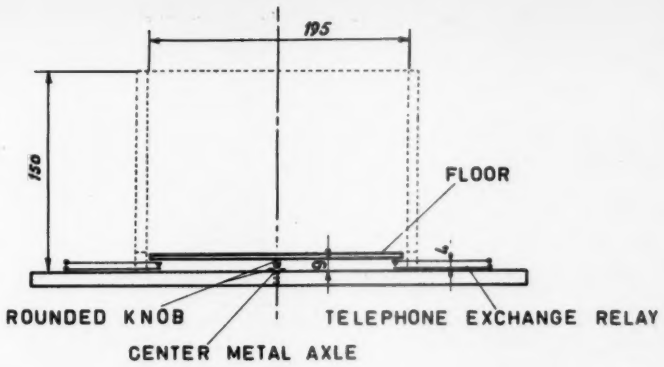

Fig. 1. General construction of the activity cage. Materials plywood, wood and glass. Recording floor of varnished hardboard. Measures in mm.

function as electrical contacts. The floor is supported in the center by a vertical metal axle which ends in a rounded knob fitting into a corresponding excavation in the supporting metal plate. Thus the floor balances on the center axle and its edges rest lightly on the contacts. As floor material varnished hardboard is used. This material is unaffected by moisture and does not become slippery. Sufficient space is left between the walls and the edges of the floor to allow free movements of the floor as well as the fall of all droppings down on the bottom of the cage.

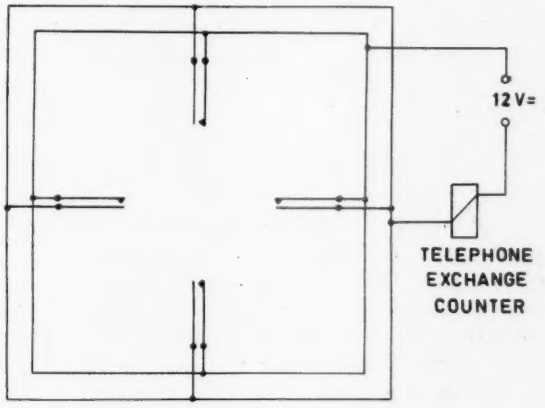

Fig. 2. Arrangement of electrical contacts (telephone exchange relays) for recording movements of floor.

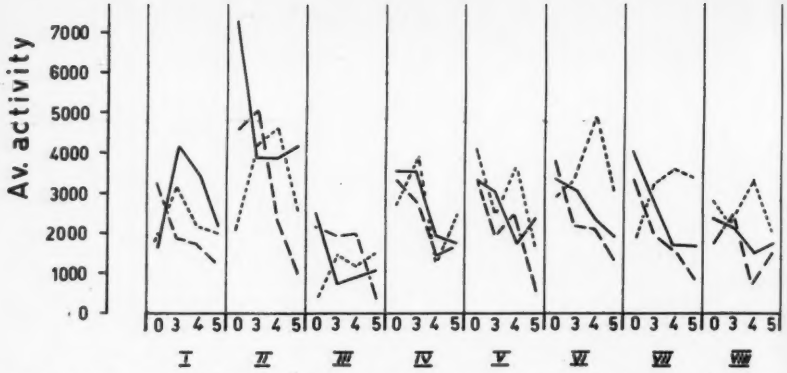

Fig. 3. Activity records of mice. Ordinate: number of impulses recorded in one hour experiment (average of group). Abscissa: roman numerals I—VIII indicate groups of 6 mice, arabic numerals indicate alcohol dose mg/g body weight. ——— initial activity record; — — — second record two weeks later; . . . . third record after four additional weeks.

The tension of the relay springs is calibrated so that a 13 g weight placed upon the edge of the floor above the relay will cause contact. Movements of an adult mouse anywhere on the floor will result in contact in some of the relays.

The arrangement of the relay contacts is shown in Fig. 2. They are connected in parallel to a telephone exchange counter (one counter for each cage) which will record the impulses from the cage. The counters are photographed with set time intervals by means of a time relay and an automatic camera and in this way a cumulative record is obtained (Fig. 3). Simultaneously the impulses are registered kymographically on paper for evaluation of type of activity, length of resting periods, exact time of activity bursts, etc. In our laboratories, a battery of six such cages is in use.

Fig. 3 illustrates the reproducibility of the activity records obtained.

The author wants to thank Mr. P. NEUENSCHWANDER for devicing the impulse recorder.

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From the Department of Physiology, University of Turku, Finland

Studies of the Effect of X-Rays on the β-Glucuronidase Activity in the Gastric Mucous Membrane and in the Liver and of its Relation to the Glucuronide Conjugation

By

K. HARTIALA, V. NÄNTÖ, U. K. RINNE and P. SAVOLA

Received 28 October 1959

Abstract

HARTIALA, K., V. NÄNTÖ, U. K. RINNE and P. SAVOLA. *Studies of the effect of X-rays on the β-glucuronidase activity in the gastric mucous membrane and in the liver and its relation to the glucuronide conjugation.* Acta physiol. scand. 1960. *49*. 65—73. — The effect of local X-irradiation of the liver and the stomach on the β-glucuronidase activity was studied in rats. In the liver it was found that with the both radiation doses, 400 r and 1,200 r, there was an initial increase in the enzyme activity which was after the first day followed by a return slightly though insignificantly below the control level. After the 3rd day a new rise greatly above the control level was present. This persisted at least 2 weeks after the irradiation.

In the gastric mucosa there was only a depression in the enzyme activity beginning already 3 hours after the irradiation. The depression continued over a period of at least 12 days. Comparison of the post-radiation β-glucuronidase activity and the glucuronide conjugation capacity of the same tissues revealed a parallelism for the gastric mucosa. In the liver the same stimulation and depression phases were also present for the both activities. Still they were not parallel so that the time relationships showed a distinct dissociation. The possible meaning of these relations are discussed.

Studies of the immediate mechanism involved in the glucuronide conjugation reactions have revealed a. o. that substances with high-energy phosphate bounds are associated with these reactions and that the formation of UDPG and UDPGA are linked to the cycle which leads to the glucuronic acid

conjugates with certain aromatic and phenolic compounds (DUTTON and STOREY 1951, 1953, SMITH and MILLS 1954). The enzymatic nature of the final transfer reaction between UDPGA and the phenol is, however, still obscure. Certain observations indicate that β-glucuronidase would play some role also in the glucuronide synthesis (FISHMAN and SIE 1956).

We have previously found in connection with studies of the effect of ionizing radiation on the hepatic and gastrointestinal glucuronide synthesis reactions that these reactions are reversibly depressed by local X-ray irradiation (HARTIALA, NÄNTÖ and RINNE 1958, 1959). In this paper we present our observations on the effect of local X-ray irradiation on the β-glucuronidase activity of the irradiated tissue and also a correlation is made of these observations to our previous results of the glucuronide conjugation reactions under the same experimental conditions.

Material and Methods

The β-glucuronidase determinations were made from the same previously reported rat-material in which the glucuronide conjugation analyses were made (HARTIALA, NÄNTÖ and RINNE 1958, 1959). Altogether 143 male rats were used (Wistar, weight range 180—220 g). The local X-ray irradiation was performed with single 400 and 1,200 r doses (185 kV X-ray machine, 10 mA, 0.5 cm Cu). The enzyme analyses were performed from the gastric mucosa specimens only in that group which recieved the 1,200 r dose.

The β-glucuronidase assay was performed according to the method described by TALALAY, FISHMAN and HUGGINS (1946) and modified by FISHMAN, SPRINGER and BRUNETTI (1948). In this method phenolphthalein-mono-β-glucuronic acid (Sigma Chemical Company) is used as the substrate. Immediately after the animals were killed a 1 per cent aqueous homogenate was made using a Potter-Elvehjelm all-glass homogenizer at + 7° C. The enzyme analyses were performed from 0.1 ml of the homogenate and duplicate determinations were made from each sample. The color intensity of the phenol liberated by the hydrolysis was measured at pH 10.45 (glycine-NaOH buffer) using Beckman DU spectrophotometer and the wavelength 540 mμ.

Results

Gastric mucosa. As can be seen from Table I and Fig. 1 a distinct reduction in the enzyme activity can be noted already 3 hours after the irradiation (the difference between the control values is significant). Between the days 5 and 12 days after the irradiation the enzyme activity had decreased still more remaining approximately at the same level between this time.

Liver. In order to obtain some information of the possible effect of the radiation dose itself the enzyme analyses were performed in two different experimental groups recieving a 400 and 1,200 r dose. In both groups (Table II and Fig. 2) an immediate strong but short postradiation increase in the enzyme

Table I. Effect of local X-ray irradiation on the β-glucuronidase activity in the gastric mucosa. Radiation dose 1,200 r

Time after irradiation	Number of animals	β—glucuronidase units/g	P
3 hours	11	1.218 ± 74	< 0.05
5 days	8	1.100 ± 39	< 0.001
12 »	9	1.069 ± 78	< 0.01
32 »	6	1.571 ± 164	> 0.05
Controls	12	1.448 ± 50	

activity was noted (the difference to the controls highly significant). This was followed after 1 day by a return slightly below the control level (not significant). The increase in enzyme activity in that group which had the smaller radiation dose was much greater; the difference between the two groups at the 12 hour point is highly significant ($P < 0.001$). This difference is not, however, significant anymore at the 1st, 3rd and 5th postradiation days. On the first day the enzyme activity values at their minimum hardly differ from the control values. On the 12th postradiation days at which the last analyses were performed, the enzyme activity in the 400 r group was approximately twice as high as in the control group. In the 1,200 r group these values are markedly less, the difference between the 400 r and 1,200 r groups on the 8th day is significant ($P < 0.005$) and on the 12th day highly significant ($P < 0.001$).

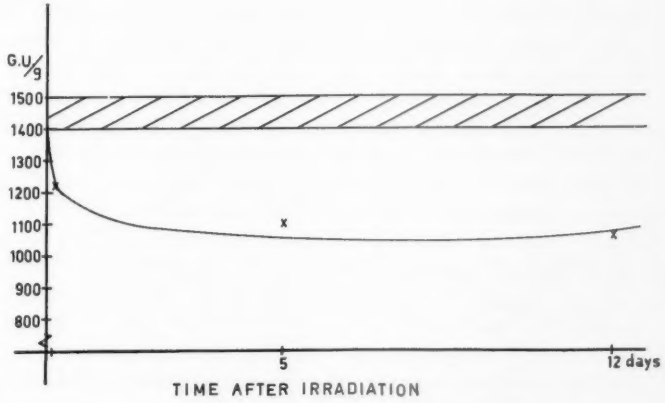

Fig. 1. Effect of 1,200 r X-ray dose on the β-glucuronidase activity of the gastric mucosa of rat (β-glucuronidase units per g wet weight tissue).

Table II. Effect of local X-ray irradiation on the β-glucuronidase activity in the liver

Time after irradiation	Group 400 r			Group 1,200 r		
	Number of animals	β—glucuronidase units/g	P	Number of animals	β—glucuronidase units/g	P
12 hours	6	5.437 ± 128	< 0.001	8	4 214 ± 103	< 0.001
1 day	8	2.840 ± 283	> 0.05	8	2 699 ± 140	> 0.05
3 days	5	2.847 ± 365	> 0·05	7	3 199 ± 154	< 0.05
5 »	5	3.772 ± 207	< 0.05	6	3 278 ± 175	> 0.05
8 »	8	5.002 ± 206	< 0.001	5	4 071 ± 309	< 0.01
12 »	6	5.698 ± 208	< 0.001	6	4 570 ± 189	< 0.001
Controls	19	3 034 ± 150				

Discussion

Usually total body irradiation, in addition to the *in vitro* experiments, has been applied in studies of the effect of ionizing radiation on certain enzyme systems. The effect has varied greatly. CARTER (1949) has claimed that in the rat spleen the activity of some enzymes (*e. g.* ribonuclease) was reversibly decreased after the irradiation whereas the peptidase activity a. o. was greatly increased. SMITH and LOW-BEER (1957) observed that the hepatic uridylic

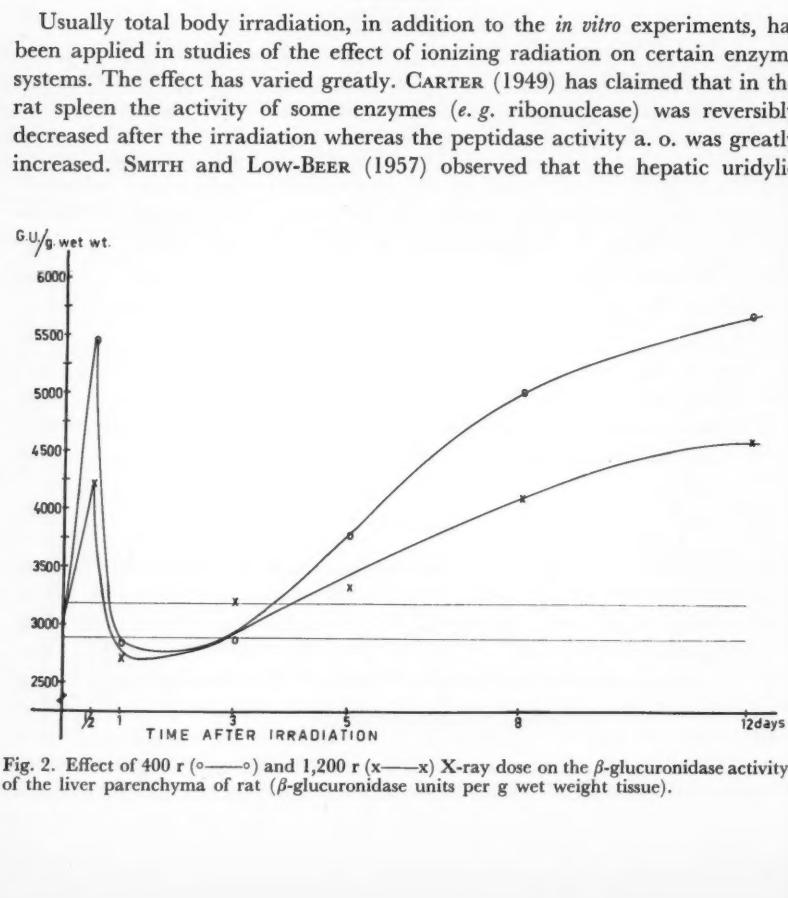

Fig. 2. Effect of 400 r (o——o) and 1,200 r (x——x) X-ray dose on the β-glucuronidase activity of the liver parenchyma of rat (β-glucuronidase units per g wet weight tissue).

acid dephosphorylase activity in rat increased after total body irradiation, this increase being greater after greater radiation doses. Under the same conditions no effect was found on some other hepatic enzyme activities.

Not much information is available of the radiation effects on the β-glucuronidase activity (ORD and STOCKEN 1953). PELLEGRINO and VILLANI (1957) have in their studies on the rat lymphatic tissue found that total body irradiation causes an immediate increase in the β-glucuronidase activity in this tissue. According to these observations a positive correlation exists between this enzyme activity and the amount of atrophy caused by the radiation.

Our own observations in which local irradiation has been applied in order to try to eliminate the probable secondary effects associated with total body irradiation, indicate that this treatment has effected the β-glucuronidase activity. This effect in the two studied tissues is, however, different. In the gastric mucosa the enzyme activity is reversibly depressed. In the liver on the other hand the actual depressing effect is lacking and in the beginning there is a short-term increase. Thereafter it returns to a level which is practically same for the both applied radiation doses and which does not significantly differ from the control level. After this a new strong increase in the activity is present on the 3rd day after the irradiation. This increase appears to extend for a considerably long time being apparent even on the 12th day after the irradiation.

MILLS, PAUL and SMITH (1953) have found in the regenerating rat liver following partial hepatectomy that the β-glucuronidase activity increases after the 4th day after the operation. This resembles somewhat the increase found in our present study.

As to the functions of β-glucuronidase in the organism some investigations consider it only as a nonspecific phenomenon associated with cellular proliferation and tissue growth processes. The enzyme would under these conditions carry hydrolytic activity (*e. g.* LEVVY 1948, KARUNAIRATNAM and LEVVY 1949, LEVVY, KERR and CAMPBELL 1948). MILLS *et al.* in their previously mentioned paper (1953) found, however, no correlation between the liver regeneration following hepatectomy and the β-glucuronidase activity but rather that the increase in the enzyme activity is associated to other metabolic processes in the tissue. The studies of FISHMAN have brought some evidence which would indicate that β-glucuronidase would participate in glucuronide conjugation syntheses (FISHMAN 1940, FISHMAN and SIE 1956, FISHMAN and GREEN 1957). It would appear from these studies that β-glucuronidase would have an important role in the metabolism of the estrogen hormones and these effects are closely related to processes of cell division and multiplication.

When the results obtained with the β-glucuronidase activity after X-ray irradiation are compared to our previous observations on the effect of same irradiation on the glucuronide conjugation capacity of the same tissues, we can find that these effects are more parallel in the case of gastric mucosa (Fig. 3). Statistically a correlation also exists between these two processes (Fig. 6).

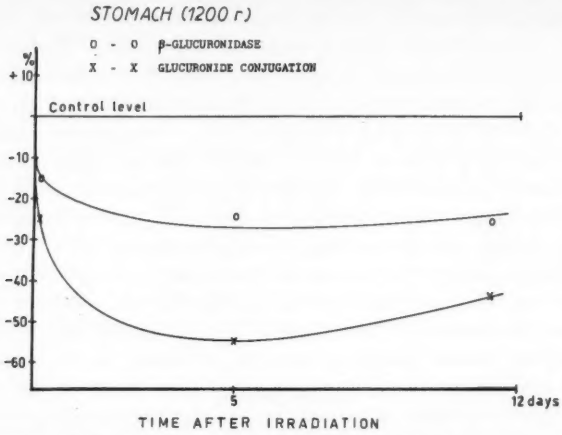

Fig. 3. Comparison of the β-glucuronidase activity (o——o) and the glucuronide conjugation capacity (x——x) of the gastric mucosa after a local 1,200 r X-ray dose.

Regarding the liver (Fig. 4 and 5) it is also noted a somewhat similar effect of the both radiation doses on the β-glucuronidase activity and glucuronide conjugation. Soon after the irradiation there is a rise in these activities, then there is rapid decrease and after few days again a new great rise. Still, some striking differences occur. A constant time lag is evident at various phases. Thus the highest point of the enzyme activity has been reached when the

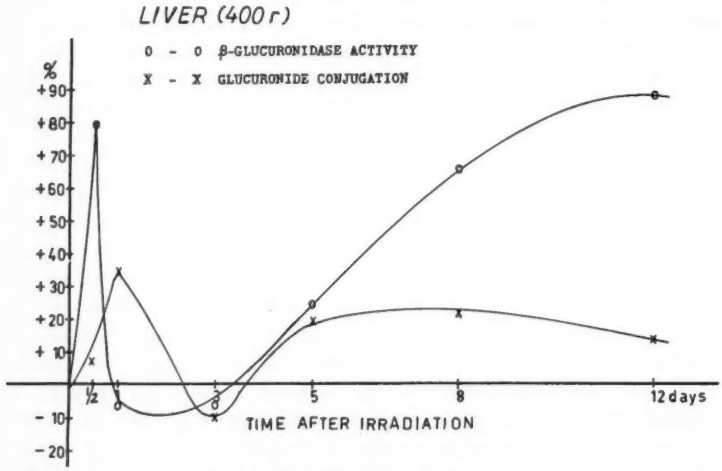

Fig. 4. Comparison of the hepatic β-glucuronidase activity (o——o) and the glucuronide conjugation capacity (x——x) after a local 400 r X-ray dose.

Fig. 5. jugatio

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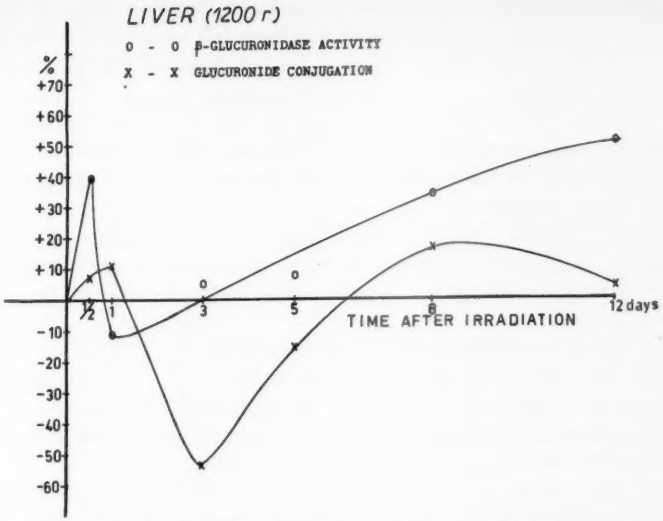

Fig. 5. Comparison of the hepatic β-glucuronidase activity (o——o) and the glucuronide conjugation capacity (x——x) after a local 1,200 r X-ray dose.

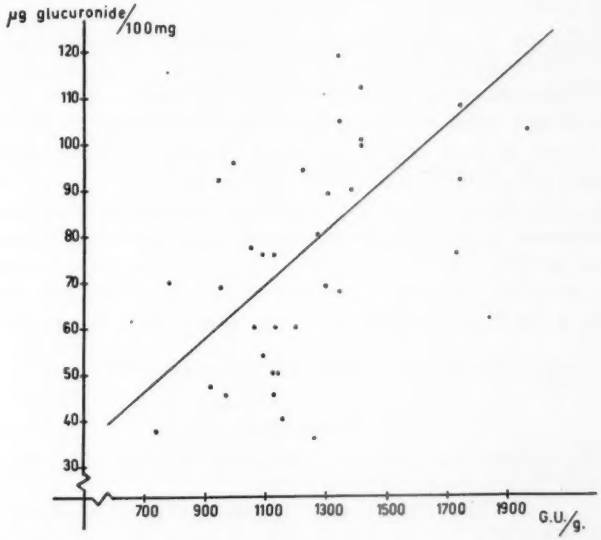

Fig. 6. Correlation between the glucuronide conjugation capacity and β-glucuronidase activity of rat gastric mucous membrane after local 1,200 r dose irradiation ($r = 0.5472$, $P < 0.01$).

conjugation activity yet only shows a very slight increase. Also at the time when the conjugation capacity is at its maximum on the first day, the enzyme activity is already at its minimum. In the 400 r group in which the conjugation capacity does not even at its lowest point decrease much below the control level, coincides the compensatory increase for the both activities on the 3rd and 5th days approximately together. With the 1,200 r radiation dose the great reduction in the conjugation capacity occurs again at a phase when the enzyme activity already has begun to rise. Furthermore it can be noted that after having reached its maximum during the compensation phase on the 8th day the conjugation capacity decreases rapidly and reaches on the 12th day the control level of intact animals whereas the β-glucuronidase activity still continues its rise.

Furthermore it is to be noted that the depressive effect of the two different radiation doses on the enzyme is at its strongest point — on the 1st and 3rd days after the irradiation — practically at the same time. Now here the 400 r dose had caused no significant depression in the conjugation while the 1,200 r dose caused a very strong reduction after the 1st day.

These observations may be interpreted so that the β-glucuronidase activity and the synthetics of glucuronides are two distinct functions and that the named enzyme plays no part in the actual conjugation processes. The problem of the physiological role of β-glucuronidase appears to be a rather complicated. This enzyme is present in various tissues, some of them being not able to carry direct glucuronide conjugation reactions (*e. g.* spleen, uterus). Here this enzyme obviously serves the purpose of liberating conjugated substances by means of hydrolysis. Whether this is necessary for the transfer of these substances into the cells or out of the cells must yet be shown. Before this problem is solved it is not possible to draw any definite conclusion of the role of the β-glucuronidase in those organs which are capable of performing both the synthesis and hydrolysis of glucuronide-compounds.

On the other hand the obvious difference between gastric mucosal and hepatic responses to the radiation treatment supports our previous observations which indicate that the sensitivity towards various experimental factors (thyroxin, cortisone, HARTIALA and HIRVONEN 1955, HARTIALA, HALME and PEKANMÄKI 1958, 1959) is different in the liver and in the gastric mucosa.

This study has been supported by a grant from The SIGRID JUSÉLIUS Stiftelse.

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From the Zoophysiological Laboratory, Department of Biological Isotope Research, University of Copenhagen, Denmark

The Kinetics of Na^{24} Flux Across Amphibian Skin and Bladder[1]

By

T. Hoshiko[2] and H. H. Ussing[3]

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Abstract

Hoshiko, T. and H. H. Ussing. *The kinetics of Na^{24} flux across amphibian skin and bladder.* Acta physiol. scand. 1960. *49.* 74—81. — The sudden addition of Na^{24} to the solution bathing one side of isolated frog skin or toad urinary bladder results in a steady increase in the labelled flux with time to a final steady state value. The build-up of the labelled flux follows a single exponential course for both influx and outflux. This is consistent with a model involving a single sodium pool in the membrane. The build-up half-times for toad urinary bladder and for frog skin averages about 2—5 minutes and is not accountable as diffusion delay through the connective tissue. The steady state flux, divided by the time constant of flux build-up, gives an estimate of the minimal amount of sodium in the pool, if only one pool is responsible for the delay. The calculated sodium pool size for urinary bladder and skin is in the same range, from 0.01 to 0.42 μeq Na/cm^2. This amount exceeds any amount imaginable as residing in the 250 Å thick basement membrane which has been proposed as the site of active transport. The pool is probably located in the epithelial cells, and the actively transported sodium passes through and not between the epithelial cells.

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[2] This work was done during the tenure of a Research Fellowship of the American Heart Association (1955—1956). Present Address: Department of Physiology, Western Reserve University School of Medicine, Cleveland 6, Ohio, U. S. A.

[3] Present Address: Institute of Biological Chemistry, University of Copenhagen.

Recently KOEFOED-JOHNSEN and USSING (1956, 1958) proposed a model to explain the nature of the frog skin potential. It is assumed that the epithelium behaves as a compartment whose boundaries against the outside and the inside bathing solutions have different permeability characteristics: The outward facing membrane is permeable to sodium and chloride but practically impermeable to potassium (and sulphate). The inward facing boundary is permeable to potassium and chloride ions but only sparingly permeable to free sodium ions. Sodium passes through this membrane primarily by way of the "sodium pump" which carries sodium from the cell to the inside bathing solution, possibly in a "forced exchange" for potassium ions.

On the basis of this model it was expected that if Na^{24} were suddenly added to the solution bathing one side of the skin, the isotopic flux or the rate of appearance of radioactivity in the opposite bathing solution would increase exponentially with time to a final steady state value. The time course of the appearance of isotope in the initially unlabelled side should involve only one exponential term, in accordance with the kinetics of such a model (SCHOFFENIELS 1957). The present experiments confirm this expectation and yield information about the minimal sodium content of the epithelium.

Methods

Skins from frogs (*Rana temporaria, R. esculenta,* and *R. oxyrhinna*) and toads (*Bufo bufo*) and toad urinary bladder were clamped between perspex chambers similar to those described previously (KOEFOED-JOHNSEN, USSING and ZERAHN 1952). The volume of Ringer's solution in each chamber including the funnels was 5 ml, and the area of membrane exposed was 2 cm^2. Ringer's solution containing NaCl 111 mM, KCl 2 mM, $CaCl_2$ 1 mM, $NaHCo_3$ 2.4 mM bathed each surface and were circulated by a stream of compressed atmospheric air. The membranes were short-circuited after mounting (USSING and ZERAHN 1951) and allowed to come to a steady state. The experiments were made at room temperature (20—24° C).

After the equilibration period (one to two hours), tracer amounts of Na^{24} were added to one of the bathing solutions and a stop watch was started. At varying intervals (1—10 min) for a total period of at least 1 hour, 500 μl samples were withdrawn from the opposite bathing solution using constriction pipettes and delivered into planchets for air drying and counting. Each sample was replaced by an equal volume of non-active Ringer with a Krogh-syringe pipette. The sampling procedure took less than 3 sec. The mixing time in the chambers was not determined but qualitative observations on dye dispersion under usual rates of gas bubbling which served to stir the solutions indicated complete mixing in less than 15 sec. An aliquot from the "hot" bathing solution served as a standard for the counting procedure. Thus the sample counts were straddled by the standard counts, obviating decay corrections.

The rates of appearance of activity (m) were calculated for each sampling period and expressed as fractions of the final steady state value (M). For each experiment, the quantity (1—m/M) was plotted against time at the midpoints of each sampling period (Fig. 1). A straight line was fitted by inspection and from it the half-time and rate constant were calculated for each experiment. In this way the time course of the isotopic flux as it attained its steady state value was determined in separate experiments for influx and for outflux.

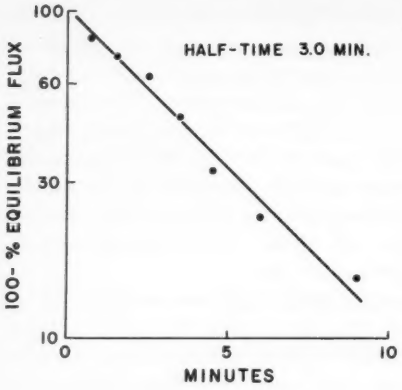

Fig. 1. Toad bladder Na^{24} influx is represented as the semi-logarithmic plot of (100- % Equilibrium flux) against time. Zero time is the time of addition of Na^{24} to the outside bathing solution.

The kinetics of washout of connective tissue sodium towards the inside bathing solution sodium was determined as follows:

Suitable pieces of unmounted abdominal skin were equilibrated for 1 hour with Na^{24}-containing Ringer. The skin was then blotted and mounted in the usual chamber. Into the chamber and reservoir on the corium side of the skin, 5 ml of inactive Ringer was suddenly added and a stopwatch started. At 15 sec intervals for the first 3 min, 500 μl samples were withdrawn and replaced with inactive solution. The solution was again sampled at 5, 10 and 15 min. The outside surface of the skin was not bathed. The cumulative washout with time was plotted for each experiment. From the initial washout rate and the final amount of sodium washed out the rate constant and half-time were calculated. The sodium content of 5 bladders was estimated by counting the bladder sodium after equilibration with sodium of known specific activity.

Results

Table I summarizes the results of 24 experiments on short-circuited isolated membranes. In all but one of these experiments the data could be fitted by a single exponential curve against time. The one exception was found in an outflux experiment done on a short-circuited frog skin where two exponentials were necessary. The data from the fast component of this experiment accounting for 63 per cent of the steady state outflux were included in Table I, but omission does not alter the final results.

The half-times for the flux build-up for all three membranes and for influx as against outflux are not significantly different and average 3.9 min. The fact that in all but one experiment of the present series only one exponential curve was necessary to describe the time course of the flux build-up would tend to indicate that diffusion delay through the connective tissue layer is not significant. If such a diffusion delay were of importance, an infinite series of exponentials would be expected.

Table I. Half-time and sodium pool content (M_i/k) from influx and outflux build-up experiments. In outflux experiments, M_i used in calculating M_i/k was obtained as the sum of the short-circuit current and M the outflux measured with Na^{24}

	Half Time (min)		M_i/k (μeq cm^2)	
	Influx	Outflux	Influx	Outflux
Frog Skin	4.0	2.5	0.049	0.055
	3.1	1.7	0.046	0.030
	3.2	4.8	0.093	0.136
Average	3.4	3.0	0.063	0.077
Toad Skin	6.0	7.4	0.210	0.135
	4.7	1.7	0.119	0.034
	4.4		0.068	
	4.8		0.106	
	5.6		0.250	
Average	5.1	4.5	0.151	0.084
Toad Bladder	5.2	2.4	0.170	0.304
	5.2	4.0	0.060	0.090
	5.4	4.2	0.042	0.418
	3.6	4.3	0.028	0.265
	3.0	0.6	0.093	0.011
	2.4		0.063	
Average	4.1	3.1	0.076	0.218

In a further attempt to assess the importance of diffusion delays in the flux build-up curve, estimates of the half-time of washout of the connective tissue layer of frog skin were made in 6 experiments. The half-time for sodium washout averaged 0.8 min (range 0.71—1.00). The total sodium washed out averaged 5.1 μeq/cm^2, (range 4.0—7.5). The use of the initial rate in this calculation may give low values but the mixing delay in the chamber may tend to give high values. In spite of these uncertainties and assuming the more unfavorable error, the build-up half-times were sufficiently large to be distinctly different from the washout half-times. These data together with the fact that one exponential curve suffices to describe the flux build-up curve indicated that the major barrier to sodium movement is not diffusion through the connective tissue layer.

The fluxes were calculated per unit area of membrane exposed. This procedure is of questionable value in the case of toad bladder. It was often observed that after dissection the toad urinary bladder contracted strongly. In mounting these bladders in the chamber, it was difficult to stretch the bladders uniformly. In addition, during the equilibration period, the previously

contracted bladder sometimes relaxed and threatened to bulge if the chamber pressures were not equal. These difficulties contribute to the variability in experiments on toad bladder.

The final column of Table I is a calculated quantity M_i/k, which is taken as a minimal estimate of the sodium pool content. M_i was obtained in the outflux experiment as the sum of the outflux and the net sodium flux calculated from the short-circuit current.

Discussion

Steady state isotope distributions have recently been used to calculate rate constants across oriented biological membranes (SCHOFFENIELS 1957, HOGBEN and GREEN 1958, and LEAF 1958). However, in frog or toad skin it is difficult to determine the specific activity of intracellular sodium since much of the activity may be lodged in the extracellular space. With the present kinetic approach it is possible to make a minimal estimate of the intracellular sodium pool and some deductions about the flux ratios across the diffusion barriers within the membrane (see Appendix).

The flux build-up delay cannot be due to diffusion delay in the connective tissue layer because: (a) the flux build-up curve can be described by a single exponential with the time and not an infinite series of exponentials as would be expected with diffusion through a finite layer; (b) the half-time for connective tissue washout is much less than the build-up half-time; and (c) there is no significant difference between the build-up half-times of skin and bladder although the toad bladder connective tissue layer is of negligible thickness compared to that of skin. Therefore the build-up half-time is not due to a diffusion delay in the connective tissue.

The true skin epithelial cell contents must lie somewhere between the present minimal pool estimates (M_i/k) and the total skin content obtained from the washout experiments here or by direct analysis by HUF, WILLS and ARRIGHI (1955). HUF's figure of 70 μeq/g wet wt. fresh skin would be 4.55 μeq/cm^2 if the wet weight figure of 65 mg/cm^2 obtained in the present study is used. This compares well with the washout figure of 5.1 μeq/cm^2, since prolonged soaking is accompanied by a steady increase in the sodium content. The frog skin sodium pool estimate from the kinetic analysis was 0.07 μeq/cm^2 or about 1.4 % of the total skin Na. If 5 % of the skin sodium were intracellular,[1]

[1] HUF, *et al.* (1955) report the average sodium content in fresh skin to be 68.7 μeq/g wet weight. Assuming chloride to be extracellular, the calculated intracellular sodium concentration was found to be negative. After eight hours of immersion in Ringer's solution containing 119 μeq/ml Na and 4.7 μeq/ml K, the skin sodium content was 92.1 μeq/g wet wt., and the calculated intracellular sodium content 10.9 μeq/g wet wt. Hence 11.8 % of the skin sodium was "intracellular". After soaking in 118 μeq/ml Na and 2.5 μeq/ml K, 4.7 % of the skin sodium was "intracellular".

the present pool estimates would be less than one third of the intracellular content. From eqn. (5) (see Appendix), the minimal correction factor for a total flux ratio of 0.1 should be roughly 1.7. Therefore the correction factor necessary for frog skin is not the minimal one and the two individual flux ratios at the inner and outer cell borders (f_i and f_o; see theoretical section) may not be equal. If the true build-up half-time in skin were smaller than the measured value due to a systematic additional delay in the connective tissue, the pool estimate would be smaller and the correction factor, larger. In the case of the bladder, the estimated pool content was roughly 10 % of the total sodium. Hence, the correction factor may not be far from the theoretical minimum of 4 and the individual flux ratios are close to equality.

The skin model of KOEFOED-JOHNSEN and USSING (1956, 1958) visualizes a large number of identical compartments in parallel containing what can be considered a single sodium pool. This assumption of a single sodium pool is in accord with the present finding that a single exponential suffices to describe the time course of Na^{24} flux across skin or bladder. Such a time course would not be expected if diffusion through the interspace between cells were the limiting step. The minimal estimate of the sodium pool size was 0.07 μeq/cm^2. This amount is much too large to be imaginable as residing in the 250 Å thick basement membrane, which has been proposed as the major barrier to sodium movement across the skin (LINDERHOLM 1954). Therefore it is concluded that the sodium pool is located in the epithelial cells and that the actively transported sodium passes through and not between the cells.

Appendix

The analysis of the kinetics of transcellular isotopic flux made by SCHOFFENIELS (1957) is directly applicable to the present case. Assuming the model depicted in Fig. 2, where fluxes instead of rate constants are used, expressions analogous to those of SCHOFFENIELS are obtained. The steady state influx (M_i) is:

$$M_i = r_2 r_4/(r_2 + r_3), \tag{1}$$

which is equivalent to SCHOFFENIELS' equation (10). His equation (7') is

$$ln \frac{(1 - m_i)}{M_i} = - \frac{(r_2 + r_3)}{A} t, \tag{2}$$

where m_i is the influx at time t which is the time elapsed since addition of label; A is the total intracellular sodium; and r_1, r_2, r_3, and r_4 are the sodium fluxes across the cell borders in the directions indicated in Fig. 2. The graph of $(1 - m/M)$ versus time on semilogarithmic paper will give a slope (k) directly, which will be identical for both the influx and outflux cases:

$$k = (r_2 + r_3)/A \tag{3}$$

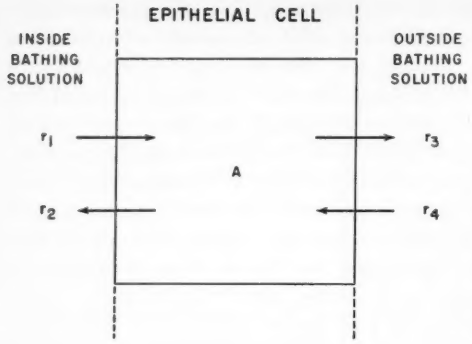

Fig. 2. Compartment model for sodium flux kinetics. The four sodium fluxes across the epithelial cell borders facing the inside and the outside bathing solutions are r_1, r_2, r_3 and r_4. The arrows indicate the directions of each flux. A is the total amount of sodium in the epithelial cell.

It is possible to obtain a minimal estimate of the size of the sodium pool involved in the flux build-up curve. From equations (1) and (3), the sodium pool (A) would be:

$$A = \frac{M_i}{k} \cdot \frac{(r_2 + r_3)^2}{r_2 r_4} \tag{4}$$

In other words, the ratio M_i/k estimates the pool (A) but equals it only when multiplied by a correction factor, $R = (r_2 + r_3)^2/r_2 r_4$. This correction factor can be expressed in terms of the flux ratios at the two borders, namely $f_i = r_1/r_2$ and $f_o = r_3/r_4$.[1]

$$\text{Then } R = f_i f_o + f_i + f_o - 1 + \frac{(1 - f_o)}{(1 - f_i)} + \frac{(1 - f_i)}{(1 - f_o)} \tag{5}$$

where $0 < (f_i f_o) < 1$. The function R can be regarded as a function of the overall flux ratio F and one of the individual ratios. Then it can be easily shown that for any given value of F, R will be a minimum when two individual flux ratios are equal, namely when $f_i = f_o = F$. For high values of F as the quotient f_i/f_o approaches 1, the limit of R is 4, while for low values of F, the limit is 1. In cases where $f_i = f_o = 1$, eqn. (5) is indeterminate and the correction factor is $R = (2 - r_i/r_o - r_o/r_i)$, where $r_i = r_1 = r_2$, and $r_o = r_3 = r_4$.

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[1] These are related to the overall flux ratio $F = M_o/M_i = (r_1 r_3)/(r_2 r_4) = f_i f_o$. For the sake of convenience, the flux ratios f_i, f_o and F as defined here are the inverse of the original definition (USSING 1949).

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From the Pharmacological Institute, University of Uppsala, Sweden

On the Binding of Zinc and Mersalyl to the Contractile Element in Muscle and the Relation to Its Relaxing Effect

By

K. A. Paul Edman

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Abstract

Edman, K. A. P. *On the binding of zinc and mersalyl to the contractile element in muscle and the relation to its relaxing effect.* Acta physiol. scand. 1960. *49*. 82—91. — In order to elucidate the functionally relevant coupling of zinc and mersalyl with the contractile element in muscle, a comparative study of their relaxing (or contraction-inhibiting) effects on glycerol-extracted rabbit psoas fibres as concerns reversibility, pH-dependence and synergism has been performed, as well as determinations of the uptake of zinc and mersalyl by the fibres after immersion in subtotal, functionally equivalent concentrations of the metals. Zinc and mersalyl have about the same affinity for the functional sites. There is additive synergism between the zinc and mersalyl effects, as tested at different pH-levels, indicating that zinc and mersalyl act by blocking the same functional groups, probably SH-groups, in the contractile element. However, while the zinc-induced effect is strongly dependent on the pH between 6.0 and 8.0, no pH-dependence at all is found for the mersalyl effect in this pH-range. Evidently zinc and mersalyl combine with the functional groups in a different way, mersalyl conceivably by simple coupling, zinc, however, only after entering into a chelate formation, in which unprotonated groups with a pK-value of about 6.7, probably imidazole groups, have to be included.

The zinc ion is able to abolish the contractility of glycerol-extracted fibres. In fibres previously contracted addition of zinc brings about complete and fully reversible relaxation (Edman 1958), if a nucleosidetriphosphate, *e. g.* adenosinetriphosphate (ATP) is present. The relaxing effect of zinc is of interest since the zinc ion might act as relaxing factor also in the living muscle cell (Edman 1959 b, 1959 c).

In a previous work (EDMAN 1959 b) the functionally relevant binding of zinc in the contractile fibres was studied. It was demonstrated that protein groups with a pK-value of 6.7, probably imidazole side chains, are involved in this coupling. It was also found that there is an additive synergism between the contractility-inhibiting effect of zinc and that obtained by mersalyl, an organic mercury compound with a high affinity for SH-groups. This finding makes it conceivable that also sulfhydryl groups partake in the functionally relevant binding of zinc to the contractile element.

The present investigation extends the earlier studies and includes, among other things, determinations of reversibility and pH-dependence of the mersalyl-induced inhibition of the contractility and an examination of the additive synergism between zinc and mersalyl at different pH-levels. The results, evaluated together with the earlier findings, will further elucidate the functional sites for zinc and mersalyl in the contractile element.

Methods

Rabbit psoas muscle extracted with glycerol as described earlier (EDMAN 1957) was used.

As medium after the glycerol-extraction the following solutions were used:

1. In the experiments performed at pH 7.3: A potassium chloride solution containing 100 mM potassium, 10 mM diethylbarbituric acid and 1 mM magnesium. This solution is referred to as "veronal buffer".

2. In experiments where pH was varied: A tris-acetate buffer solution containing 100 mM potassium chloride, 10 mM acetic acid and Tris (tris/hydroxymethyl/aminomethane) in a concentration sufficient for the desired pH.

$ZnCl_2$, pro analysi, E. Merck, mersalyl according to Ph. S. XI and Na_2H_2ATP, crystalline, Pabst were used. For analysis of the ATP preparation, lot. no. 109 A, used in the studies of zinc and mersalyl uptake, see EDMAN (1959 a). The ATP preparation, lot. no. 116 A, used in the rest of the work contains, according to an analysis performed as described (EDMAN 1957) 1.63 mmoles nucleotide per gram substance and has the following composition: ATP 96.5 percent, ADP 1.8 percent and AMP 0.9 percent; no nonadenine impurity was found. All other chemicals used were of analytical purity. The water was redistilled in borosilicate glass distillers. The concentrations given refer to the total concentration in the bath, if not otherwise stated. For the calculation of free zinc the same stability constants (log K) were used as earlier (EDMAN 1959 a), viz. 4.00 for the MgATP complex and 4.76 for the ZnATP complex.

I. *Contraction experiments.*

The technique for strict isometric recording (EDMAN 1957) was used. The deviation from ideal isometry was < 0.2 percent of the fibre length. Before the start of the experiment the prepared fibre bundles (cross section 84—131 $\times$ 154—224 μ) were, if not otherwise stated, immersed for 60 min in a Petri dish containing the test solution without ATP. The contraction, induced by 0.4 mM ATP, was recorded for 5 min, when constant tension had been attained. The initial tension applied to the fibre bundles was 15 mg. The experiments were performed at room temperature, 20—22°.

II. *Determination of the uptake of zinc and mersalyl by the extracted muscle tissue.*

The glycerol-extracted muscle was ground to a pulp in veronal buffer in an agate mortar. The ground muscle mass consisted of undifferentiated brei, single fibres and fragments of fibre bundles. The muscle tissue (about 1.5 g wet weight) was then washed twice in veronal buffer (about 100 ml) by gentle stirring for 15 min followed by 30 min centrifugation (3,500 rpm). For each experiment a quantity of the washed muscle pulp corresponding to about 0.02 g dry weight was used. This was incubated in a sealed borosilicate glass flask containing 500 ml test solution for 30 min with gentle shaking. During incubation the bath solutions did not lose more than 2 percent of the test substances. For the composition of the test solution in the different experiments, see Results. One sample of the muscle mass was analyzed for zinc and mercury immediately after pulpfaction and washing.

All vessels used in these experiments were made of borosilicate glass except the quartz glass flask used for combustion of muscle tissue (see below). All vessels were washed with 6 M redistilled HCl and water before use.

Determination of dry weight and nitrogen content.

The muscle tissue was dried to constant weight at + 105° C. Nitrogen content was determined by means of micro-Kjeldahl technique.

Analysis of zinc and mercury in muscle tissue.

The organic matter was first destroyed by means of wet combustion with HNO_3–H_2SO_4–$HClO_4$. Zinc and mercury were determined colorimetrically (Zeiss "Elko") after extraction with dithizone-carbontetrachloride according to SANDELL (1950). Extraction of mercury was performed at pH 0, extraction of zinc after the pH had been adjusted to 4.75 with potassium hydroxide and sodium acetate-acetic acid buffer. The addition of sodium thiosulphate was omitted.

Results

1. *pH-dependence of the contraction-inhibiting effect of mersalyl.*

As demonstrated in an earlier work (EDMAN 1959 b) the contraction-inhibiting effect of zinc in glycerol-extracted muscle fibres is strongly dependent upon the hydrogen concentration in the pH range 6.0—8.0. It is the aim of the present experiments to determine whether a similar pH-dependence also exists for the contraction-inhibiting effect induced by mersalyl. The bath concentration of mersalyl necessary for an almost complete inhibition (89 ± 1 percent) of the contraction induced by 0.4 mM ATP in the presence of 1 mM magnesium at different pH-levels was determined. The concentration of mersalyl required was first preliminarily tested and then verified by four pairs of experiments, each pair consisting of one test (with mersalyl) and one control (without mersalyl). The results are summarized in Fig. 1, which, for comparison, also shows the pH-dependence of the zinc effect (EDMAN 1959 b).

It is evident from Fig. 1 that the contraction-inhibiting effect of mersalyl is not significantly dependent upon the hydrogen concentration between pH 6.0 and pH 8.5, *i. e.* practically the same concentration of mersalyl is required for obtaining a definite inhibiting effect at the different pH-levels examined.

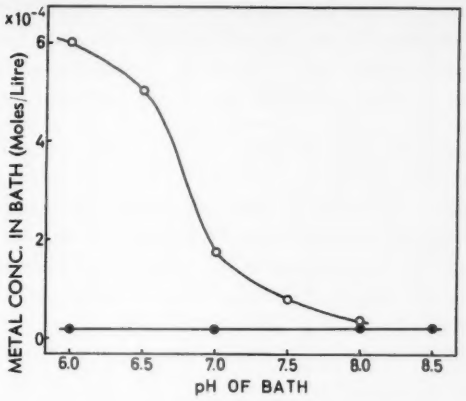

Fig. 1. Relation between pH and total bath concentration of mersalyl (filled circles) and zinc (open circles), necessary for a definite inhibition of the final isometric tension of glycerol-extracted fibre bundles contracted by 0.4 mM ATP.

The inhibition comprised 89 percent of the final tension in the mersalyl experiments and 97 percent in the zinc experiments.

Medium: Tris-acetate buffer solution containing 1 mM magnesium. Each point represents 4 tests and 4 controls.

This is in striking contrast to the situation with zinc where the contraction-inhibiting effect is strongly pH-dependent. The pH-dependence curve for the zinc effect has the form of a reversed S with the inflexion at pH 6.7.

The pH-dependence curves demonstrated in Fig. 1 illustrate approximately the inverse pH-dependence of the coupling of mersalyl and zinc to the *functional* sites in the contractile element. It is improbable that the results have been markedly influenced by complex formation of zinc and mersalyl with ATP and the buffer substances (Tris and acetate) in the solution as is evident from the following facts:

The stability constant of the zinc-ATP complex ($10^{4.76}$) is unchanged over the pH-interval tested according to the titration curves presented by WEITZEL and SPEHR (1958). The complex formation between zinc and acetate (MYRBÄCK 1955) cannot be assumed to be markedly modified by a change in pH between 6.0 and 8.0 since the pK_a for acetate is 4.76. The ability of Tris to complex zinc is insignificant (NEURATH 1955). In fact, the effect of a pH-shift from 6.0 to 8.0 on the complexing of zinc by acetate and Tris would, if anything, be expected to cause an increased need for zinc at the higher pH, in contradiction to the experimental finding.

It is uncertain if mersalyl is at all complexed by ATP or the buffer substances in the solution. In any case, the finding of a constant need for mersalyl over the large pH-interval examined makes it improbable that there is any significant pH-dependence of the complexing of mersalyl in the solution.

Thus, within the pH-interval examined, there is no significant pH-dependence of the functionally relevant binding of mersalyl to the contractile element in contrast to the marked pH-dependence existing for the functional binding of zinc. This difference is not to be explained by greater affinity of mersalyl for the functional sites, for, as is evident from the present results (see right part of Fig. 1 and Results 4), the affinity of mersalyl for the functional sites is of the same order of magnitude as that of zinc. The results will be interpreted under Discussion.

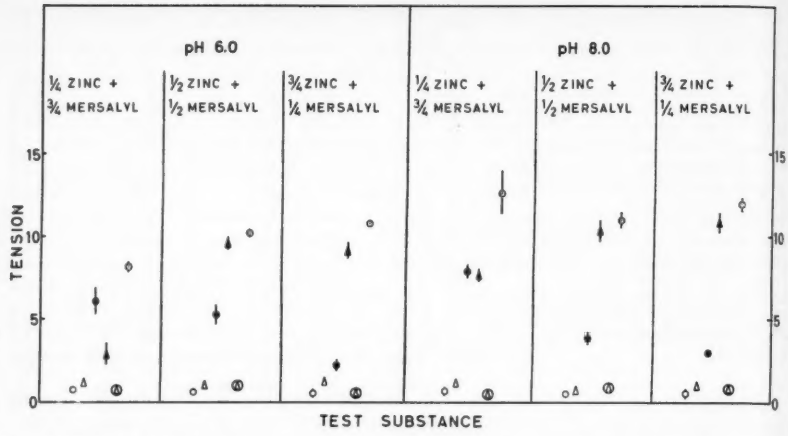

Fig. 2. Additive effects of zinc and mersalyl on the isometric contraction of glycerol-extracted fibre bundles.

Medium: Tris-acetate buffer solution containing 1 mM magnesium. Ordinate: Tension in mg per 100 μ fibre bundle circumference. Abscissa: Reading from left to right, test runs are temporarily placed.

Contraction induced by 0.4 mM ATP in the presence of:

○ : full concentration of zinc alone (at pH 6.0: 0.6 mM, at pH 8.0: 0.0375 mM),
△ : full concentration of mersalyl alone (at pH 6.0 and pH 8.0: 0.022 mM),
(△): partial concentration (1/4, 1/2 or 3/4) of zinc + partial concentration (3/4, 1/2 or 1/4) of mersalyl,
● : partial concentration of zinc alone,
▲ : partial concentration of mersalyl alone,
⊙ : control without zinc and mersalyl.

Each symbol represents the mean of 3 experiments. The standard error of the mean is indicated with a bar if it exceeds the symbol size. Note that points ○, △ and (△) all yield a tension of roughly the same magnitude.

2. *The additive synergism between the effects of zinc and mersalyl.*

As demonstrated previously (EDMAN 1959 b) there is an additive synergism between the contraction-inhibiting effects of zinc and mersalyl, a finding which indicates that zinc and mersalyl probably act on the same functional sites in the protein. The examination was performed at pH 7.3. In view of the quite different pH-dependence of the effects of mersalyl and zinc, as just described, it is of interest to know whether the additive synergism exists only at the hydrogen ion concentration originally examined, pH 7.3, or holds true also at other pH-levels.

Fig. 2 shows the results obtained at pH 6.0 and 8.0. The contraction was induced by 0.4 mM ATP. The concentrations of zinc alone and mersalyl alone needed for obtaining a definite tension, an almost complete inhibition of the contraction, were first determined. These concentrations are called "full concentrations". In accordance with the foregoing (Results 1), the full con-

centration of mersalyl is the same at pH 6.0 and pH 8.0, while the full concentration of zinc is considerably higher at pH 6.0 than at pH 8.0 (see text to Fig. 2). The tension with mersalyl and zinc in the following combinations were then determined, the concentrations stated as parts of the full concentrations: 3/4 zinc + 1/4 mersalyl, 1/2 zinc + 1/2 mersalyl and 1/4 zinc + + 3/4 mersalyl. The tension obtained with zinc and mersalyl individually in these partial concentrations was also determined as was the tension without zinc and mersalyl. The investigation was performed with fibre bundles from the same psoas muscle preparation and was carried out in series of experiments as follows: 1. zinc, full concentration, 2. mersalyl, full concentration, 3. zinc, partial concentration, 4. mersalyl, partial concentration, 5. zinc, partial concentration + mersalyl, partial concentration, 6. control without zinc and mersalyl. The experiments in each such series were carried out in immediate succession.

The coupling of zinc and mersalyl is proportional to the concentrations of free zinc and mersalyl in the solutions. For the evaluation of the experiments it is therefore essential that the proportions of free zinc and free mersalyl are roughly constant at the different combinations studied. This is also the case. Even if only complexing of zinc by ATP and not by the buffer substances in the solutions were assumed, the relation between free zinc and total zinc would be practically constant, 0.25—0.27, in the experiments at pH 8.0 and would only deviate between 0.33 and 0.56 in the experiments at pH 6.0. If mersalyl is complexed in the solution, the proportion of free mersalyl would similarly be constant because of the low mersalyl concentrations used at both the pH-levels examined.

As is evident from Fig. 2, the same tension as that obtained with zinc alone and mersalyl alone in full concentrations is produced with all the combinations of zinc and mersalyl studied at both pH-levels. Thus, the additive synergism between the zinc and mersalyl effects previously demonstrated at pH 7.3 (EDMAN 1959 b) also exists at pH 6.0 and pH 8.0.

3. *Incubation time required for reaching constant inhibiting effect of mersalyl in the fibres, and the reversibility of the mersalyl effect.*

In order to attain full effect of 0.022 mM mersalyl, *i.e.* an almost complete inhibition of the contractility of the fibres, 30 minutes' incubation of the fibres in the mersalyl bath is sufficient. Immersion for a further 30 min does not augment the effect.

It is possible by simple washing of the fibres in mersalyl-free buffer solution to restore the contractility previously abolished by mersalyl. The contractility is to a great part regained after 30 minutes' washing of a fibre bundle, which has been pretreated for 60 min with 0.022 mM mersalyl. About 4 hours' washing in mersalyl-free solution is needed, however, to restore the contractility completely. It may be noted for comparison, that only 20—30 minutes' washing in ordinary buffer solution is needed in order to completely reverse the contraction-inhibition induced by zinc (EDMAN 1958).

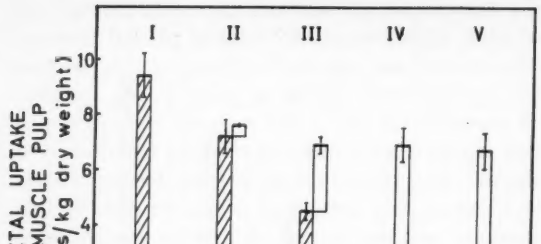

Fig. 3. Uptake of zinc and mersalyl by glycerol-extracted tissue.
Medium: Veronal buffer solution with pH 7.3 containing 1 mM magnesium, 0.4 mM ATP and:
0.025 mM zinc, full concentration (series I),
0.022 mM mersalyl, full concentration (series V),
partial concentration (3/4, 1/2 or 1/4) of zinc + partial concentration (1/4, 1/2 or 3/4) of mersalyl (series II, III and IV).
Shaded columns: zinc.
Unshaded columns: mersalyl.
Each column represents the mean of 5 experiments. The standard error of the means is indicated with a vertical bar.

Nitrogen content: 14.2 ± 0.1 percent of dry weight.

4. *Uptake of zinc and mersalyl by glycerol-extracted tissue.*

The following experiments were performed to investigate the uptake of zinc and mersalyl by glycerol-extracted fibres from bath solutions containing zinc and mersalyl in different combinations, all solutions being *functionally equivalent.* The veronal buffer solution (100 mM potassium and 1 mM magnesium) was used, and ATP in 0.4 mM concentration was consistently present. The following combinations of zinc and mersalyl in the bath were studied:

1. 0.025 mM zinc (full concentration).
2. 0.022 mM mersalyl (full concentration).
3. 1/4 concentration of zinc (0.00625 mM) + 3/4 concentration of mersalyl (0.0165 mM).
4. 1/2 concentration of zinc (0.0125 mM) + 1/2 concentration of mersalyl (0.011 mM).
5. 3/4 concentration of zinc (0.0188 mM) + 1/4 concentration of mersalyl (0.0055 mM).

As was demonstrated earlier (EDMAN 1959 b), the same effect, an almost total inhibition of the ATP-induced contraction, was obtained with all five combinations of zinc and mersalyl. The fact that the contraction-inhibiting effect is *subtotal* means that the fraction of the total uptake bound to functionally irrelevant sites in the protein is minimized.

The concentrations of zinc needed for the desired effect are about 4 times lower when veronal buffer solution is used as medium than when Tris-acetate buffer is used. For instance, about 0.1 mM zinc alone is needed for 97 percent inhibition of the contraction with the Tris-acetate buffer (see Fig. 1) as compared with 0.025 mM in these

experiments with the veronal buffer as medium. This difference is probably due to more pronounced complexing of zinc in the Tris-acetate buffer solution by the acetate ions (Cf. MYRBÄCK 1955).

Fig. 3 shows that in order to attain a definite effect of zinc and mersalyl about the same total number of moles have to be taken up in both cases. Moreover, the sum of the moles of zinc and mersalyl bound to the extracted tissue is about the same with all the functionally equivalent combinations of zinc and mersalyl studied, *i. e.* the effect is unchanged if one component, *e. g.* zinc, is replaced by uptake of the same molar amount of the other one. Thus, with regard to the contraction-inhibiting effect the uptake of a certain amount of zinc by the muscle tissue is equivalent to the uptake of an equimolar amount of mersalyl. A tendency for greater uptake of zinc by the muscle seems to exist, however. This might be an expression of a greater tendency for zinc to be bound to functionally irrelevant sites in the fibre protein.

Discussion

The finding of an additive synergism between the contraction-inhibiting effects of zinc and mersalyl makes it probable that in both cases the same functional groups in the contractile element are involved. In view of the very high affinity of mercury for SH-groups ($K > 10^{20}$) as demonstrated with small molecules (STRICKS, KOLTHOFF and HEYNDRICKX 1954, GURD and WILCOX 1956) it is probable that the mersalyl-induced contraction-inhibition is due to a blocking of SH-groups in the contractile element. This would mean, in accordance with the foregoing, that also the zinc-induced inhibition of the contraction is probably due to blocking of SH-groups.

The different pH-dependence of the effects of zinc and mersalyl cannot be explained by greater affinity of mersalyl for the functional sites, since, as demonstrated in the present work, zinc and mersalyl have about the same affinity for the functional sites in the contractile element (see Fig. 3 and right part of Fig. 1). The discrepancy in the pH-dependence therefore makes evident that zinc and mersalyl combine with the functional groups in a different way. The finding that the mersalyl effect does not have any pH-dependence between pH 6.0 and 8.5 is consistent with an involvement of only sulfhydryl groups. However, the pronounced pH-dependence of the zinc effect between pH 6.0 and 8.0 suggests that in the functionally relevant coupling of *zinc* with the contractile protein groups other than sulfhydryls are involved, viz. groups having a pK-value of ~ 6.7. According to the hypotheses advanced by CECIL (1950), BENESCH and BENESCH (1953) and MYRBÄCK and WILLSTAEDT (1958), the pH-dependence of a metal effect could result from a change in the accessibility of the SH-groups for the metal brought about by a change in the degree of protonation of adjacent groups, *i. e.* a masking of the SH-groups by the formation of a hydrogen bond from a neighboring, protonated group. However,

such a mechanism could not reasonably explain the different pH-dependence of the zinc and mersalyl effects described in the present work. As pointed out, the affinity of mersalyl for the functional sites is of the same order of magnitude as that of zinc, and a pH-induced masking of the SH-groups should therefore be expected to modify not only the effect of zinc but also the effect of mersalyl. Hence, it remains as probable that zinc, in order to block the functional SH-groups in the contractile element must enter into a chelate formation, which includes, in addition to the SH-group, also an unprotonated group with a pK-value of about 6.7. The fact that a subtotal zinc-induced inhibition of the contraction can be completely abolished by a decrease in pH from 8.0 to 7.0 (Fig. 3, EDMAN 1959 b) further supports the assumption that unprotonated groups other than sulfhydryls must also be involved. It is conceivable that the additional groups needed for the functionally relevant binding of zinc are imidazole groups, the pK-value of which is about 7 under the experimental conditions used (For references, see EDMAN 1959 b, page 225).

The results obtained thus indicate: 1. that blocking of the same functional groups, probably sulfhydryl groups, is responsible for the contraction-inhibiting effects of zinc as well as of mersalyl, and 2. that zinc in order to block these functional groups also needs involvement of other groups (pK 6.7) in unprotonated form, conceivably imidazole groups.

The additive synergism between the zinc and mersalyl effects makes it probable, as stated above, that the two agents act on the same functional groups in the protein but does not presuppose that zinc and mersalyl must be bound to the functional groups in equimolar amounts in order to induce a definite effect. The metal analyses presented in this work were performed with metal concentrations giving a definite, *subtotal* contraction-inhibiting effect, by this means bringing the fraction of the uptake of the metals bound to unspecific sites to a minimum. As shown by these experiments the uptake of a certain amount of zinc is functionally equivalent to the uptake of an equimolar amount of mersalyl. Definite conclusions as to the equivalence of zinc and mersalyl when bound to the functional sites cannot yet be drawn, since the functionally relevant uptake of the metals might be only a small fraction of the total uptake determined. The results support the assumption, however, that zinc and mersalyl can replace each other mole per mole in their blocking of the functional groups.

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From the Department of Physiology, University of Turku, Finland

Inhibition of Cinchophen Ulcer in Chicks with Boiled Potassium Hydrogen Saccharate

By

K. HARTIALA and I. HÄKKINEN

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Abstract

HARTIALA, K. and I. HÄKKINEN. *Inhibition of cinchophen ulcer in chicks with boiled potassium hydrogen saccharate.* Acta physiol. scand. 1960. *49.* 92—96. — Simultaneous feeding of saccharic acid lactone ($1^1/_2$ % of the diet) with cinchophen prevented completely the usual gastric ulcer formation up to the 10th day at which time the control group treated only with cinchophen had an ulcer in 60 per cent of the cases. At the 14th day the control group had ulcers in 85 per cent, whereas the saccharic acid treated group had ulcers and erosions in 55 per cent. Saccharic acid is known as a β-glucuronidase enzyme inhibitor. The results are discussed in light of the possible relationship of the local glucuronide conjugation reactions and cinchophen ulcer mechanisms.

Cinchophen is known as a reliable tool for production of gastric and duodenal ulcers in certain animal species (CHURCHILL and VAN WAGONER 1936). Dogs have been considered most suitable for this type of experimental ulceration. CHENEY (1940) has demonstrated this same phenomenon in chicks.

In connection of studies on the mechanism of the cinchophen ulcer attention has been drawn to the possible relationship of the newly discovered gastrointestinal glucuronide conjugation processes (HARTIALA 1954, 1955) and the inhibition of the mucous secretion by the duodenal glands upon cinchophen feeding in dogs (HARTIALA, GROSSMAN and IVY 1950). On the other hand although much progress has been made in the exploration of the biochemical machinery which carries the glucuronide synthesis reactions, the final phase in the glucuronide formation and particularly nature of the enzyme catalyst, UDP-transglucuronylase, which mediates the glucuronyl transfer to the sub-

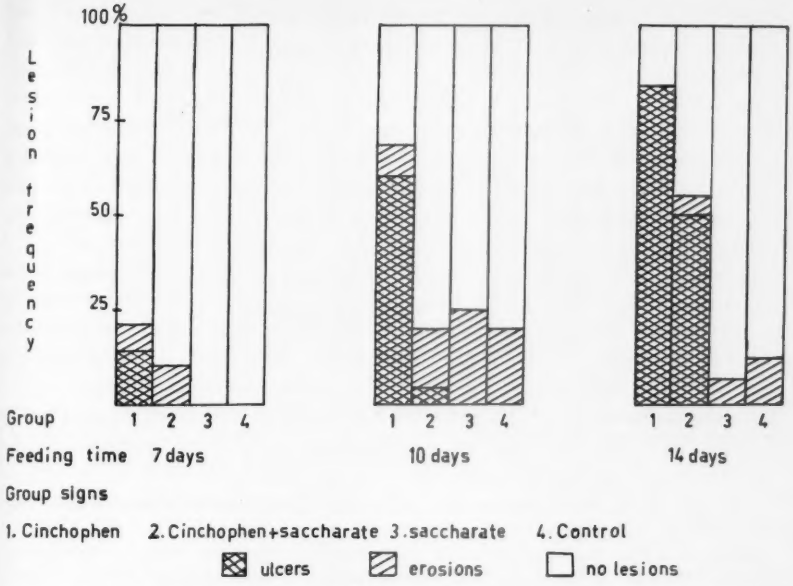

Fig. 1. Effect of saccharate on the cinchophen provoked gastric ulcer and lesion frequency in chicks.

strates, is not quite clear yet. The physiological meaning of the β-glucuronidase in this connection seems still to be a matter of great interest. The present studies were undertaken in order to find out whether the saccharic acid lactone, a substance known to be an inhibitor for the β-glucuronidase enzyme had any effect on the cinchophen ulcer production.

Material and Methods

New-born chicks were used for the purpose mainly because large experimental series can be used and also because the ulcers formed in this animal by cinchophen are very easily detectable in the muscular part of the stomach. Administration both of cinchophen and the enzyme inhibitor in the diet is very easy. The material comprises 440 chicks which were divided into 4 experimental groups. Group 1 received the basic diet (9 parts of dried fish powder and 1 part dried grass powder, water ad libitum[1]. Group 2 received cinchophen (Merck) added into the diet (1.5 per cent). Group 3 received both 1.5 per cent saccharate and 1.5 per cent cinchophen in their diet. The acidified saccharate (Merck, potassium hydrogen saccharate) was boiled under reflux over 30 min in order to transfer it to its more potent lactone form. Group 4 received only the potassium saccharate in their diet.

[1] The authors are greatly indebted to the firm Turun Muna for the free food supply for our experiments.

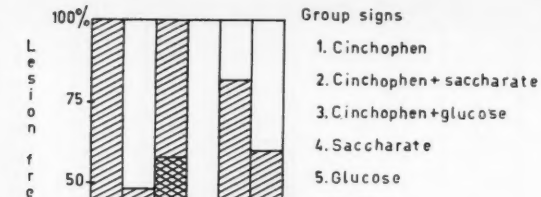

Fig. 2. Comparison of the effect of saccharate and glucose on cinchophen provoked gastric ulcer and lesion frequency in chicks.

After brought to the laboratory the 2 days old chicks were kept on the basic diet over a period of 6 days before the experiments were begun.

During this time the changes in the environmental conditions had eliminated some 30 per cent of the original amount. No spontaneous deaths occurred after this time. They were then separated into the described groups; the saccharate feeding was started 2 days before the cinchophen treatment was given. Groups 2 and 3 were sacrificed (by a blow on the head) on the 7th, 10th, and 14th day after the beginning of the cinchophen feeding. The rest of the animals were killed on the 14th day.

After the animals were killed the stomachs were exposed and opened. The ulcers were always noted in the muscular part of the stomach as described by Cheney. The gastric lesions were either erosions, deep crater-like ulcers or perforations in the same area. Following categories were used in the classification: those which showed some symptoms, either erosions or ulcer and those which were completely without symptoms.

Results

In group 2 which originally comprised 45 chicks, 5 died during the feeding. These are excluded from the material since they died before the observation dates.

From group 3 including originally 74 animals, 62 survived for the final observations. From the control groups 1 and 4, 18/20 and 22/25 survived up to the end of the experimentation. It must be pointed out that none of the animals who succumbed during the test showed sign of ulcers.

The results are graphically illustrated by Fig. 1.

They clearly indicate that the simultaneous application of the saccharate reduced both the ulcer and erosion frequency provoked by cinchophen. During the first 7 days period there were no ulcers in the saccharate plus cinchophen group whereas cinchophen alone had caused ulcers in 15 per cent of the chicks.

On the 10th day the effect is more distinct. Cinchophen alone caused ulcers in 60 per cent whereas the saccharate had reduced this frequency to 4 per cent. The difference in the total lesion frequency is also striking: 70 per cent against 10 per cent. Continued feeding of cinchophen over a period of 10 days had raised the ulcer frequency to 80 per cent at which time the ulcer frequency in the saccharate treated group was yet only 50 per cent. It is interesting to note that at this time practically all of the lesions in both groups were ulcers. No spontaneous ulcers had developed during this time in the control groups.

Further experiments were then performed in order to find out whether the apparent inhibiting effect produced by the saccharic acid lactone was due to changes in the caloric intake of the animals. In these studies glucose in equal amount ($1^1/_2$ %) was added to the diet of two of the groups, the other receiving this together with cinchophen. The feeding period in these series was 10 days.

The results are illustrated in Fig. 2. Here again the saccharate given together with cinchophen completely prevented the ulcer production and reduced the total lesion frequency to 50 %. On the other hand when saccharate was replaced by glucose the ulcer and lesion frequency was the same or even higher as when cinchophen was given alone.

Discussion

These results indicate that the interfering with the glucuronide machinery in connection with the experimental provocation of cinchophen ulcer, markedly alters the effect of the ulcerogenic substance. These results may be considered as a further support of our previous concept of the relation between the cinchophen ulcerogenic effect and the local glucuronide synthetic function in the gastrointestinal mucous membrane (HARTIALA 1954, 1955). This takes into consideration the concept of competitive functions in the mucous membrane. The mucoprotein (mucus) synthesis and secretion on the one hand and the glucuronide conjugation synthesis on the other hand are considered as such competitive functions. Loading of the latter function *e. g.* with cinchophen leads to an increased glucuronide synthesis with the cost of the mucoprotein production. This has been demonstrated in our earlier work (HARTIALA, GROSSMAN and IVY 1949). Whether this is due to the dependence of these two functions on the same enzyme machinery in the UDPG—UDPGA cycle or UDP-transglucuronylase activity is not yet quite clear. Still more obscure is the role of β-glucuronidase in this connection. It is obvious that this enzyme does not carry the transferase function between the phenol and UDPGA *e. g.* (DUTTON 1959). Before the physiological role of β-glucuronidase in the mucous membrane is clear, it is also too early to draw definite conclusions of the actual mechanism underlying our inhibiting effect demonstrated by the saccharic acid lactone on the cinchophen erosions. The action of saccharic acid lactone itself is a substrate competitive for the β-glucuronidase (LEVVY 1952, BERN-

FELD, JACOBSON and BERNFELD 1957). The theoretical end result of its effect in the conjugation machinery would be an increase in the conjugate by prevention of its hydrolysis. Simultaneous feeding of this inhibitor with another glucurogenic substance (phenolphthalein), however, does not seem to lead to an increased output of the phenolphthalein glucuronide but to a reduction in the free phenolphthalein in the urine (PEKANMÄKI and HARTIALA 1960). These fragments of experimental evidence do not yet, however, suffice to explain why this could lead to a diminished lesion provocation. Perfusion studies now under progress are hoped to throw some more light on these relationships.

This study has been supported by the Sigrid Jusélius Stiftelse.

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